Gene Expression and Cytokine Pattern of Pulmonary Tuberculosis Patients and their Contacts in Ethiopia

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Declaration

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List of Abbreviations

ABR	Active BCR (breakpoint cluster region) related
Ad	Adeno virus
Ag85A	Antigen 85 A
Ag85B	Antigen 85B
AFB	Acid fast bacilli
AIDS	Acquired Immunodeficiency Syndrome
APCs	Antigen presenting cells
ASNA1	ATP-binding arsenite transporter
ATP5G1	Mitochondrial ATP synthase
AUC	Area under curve
B2M	Beta-2 microglobulin
BCG	Bacillus Calmette Guerin
Bcl2	B cell lymphoma 2
BLR1	Burkitt lymphoma receptor 1
BPI	Bactricidal/permeability increasing
CARD9	Caspase activation and recruitment domain
CAS	Central Asian
C14orf2	chromosome 14 open reading frame 2
CCL /CXC	Chemokine ligand
CD	Cluster of Differentiation
CR	Complement receptor
CTLA4	Cytotoxic T-Lymphocyte Antigen 4
DC	Dendritic cells
DC-SIGN	DC-specific intercellular adhesion molecule- 3-grabbing nonintegrin
DNA	Deoxyribo nucleic acid
DR	Direct repeat
EAI	East African-Indian
EGF	Epidermal Growth Factor
ESAT-6	Early secretory antigen 6
FcR	Fc receptor
FcγR1A	Fc gamma receptor 1 A
FOXP3	Forkhead box P3
FPR1	Formyl peptide receptor 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDA	General discriminatory analysis
GUSB	Beta-glucuronidase
HBCs	High burden countries
HHCs	Healthy household contacts
HIV	Human Immunodeficiency Virus
ICs	Index cases
IFN-γ	Interferon gamma
IGRA	Interferon Gamma Release Assays
IκB	inhibitor of kappa B

IL	Interleukin
IL -1α	Interleukin 1 alpha
IL- 7R	Interleukin 7 receptor
IL-12p40	Interleukin 12 polypeptide 40
IL-1β	Interleukin 1 beta
IL-4 • 2	IL 4 delta2
IRAK	Interleukin 1 Receptor associated kinase
KIAA2013	hypothetical protein KIAA2013
KO	Knock out
LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
LSP	Long Sequence Polymorphisms
LTF	Lactoferrin
LY6G6D	lymphocyte antigen 6 complex, locus G6D
MAP	Mitogen-activated protein
MARCO	Macrophage receptor with collagenous structure
MCP 1	Monocyte chemoattractant protein 1
MCP 2	Monocyte chemoattractant protein 2
MCP 3	Monocyte chemoattractant protein 3
MDR	Multi-drug resistance
MHC	Major histocompatibility complex
MIG	Monokine induced by gamma interferon
MIP 1β	Macrophage Inflammatory protein 1 beta
MIRU-VNTR	Mycobacterial Interspersed Repetitive Units - Variable Number Tandem Repeats
MMLV	Moloney Murine Leukemia Virus Reverse Transcriptase
MØ	Macrophage
MPP9	Matrix metalloproteinases 9
MR	Mannose receptor
MVA	Modified vaccinia virus Ankara
MyD88	Myeloid differentiation factor 88
NFKB	Nuclear Factor Kappa B
NK	Natural killer
NK-T	Natural killer T cells
NO	Nitric oxide
NOD2	Nucleotide oligomerization domain 2
NBOS2	Nitric oxide synthase 2
NCAM1	Neural cell adhesion molecule 1
NOLA3	Nucleolar protein family A, member 3
NOS2	Nitric oxide synthase
PBMC	Periphal blood mononuclear cells
PCR	Polymerase chain reaction
Pfo	perfringolysin
PPD	Purified protein derivative
PRR	Pattern recognition receptor
QFT-GIT	Quantiferon gold in tube test
RAB33A	Ras-associated GTPase 33A

RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
rBCG	Recombinant Bacillus Calmetti Guerin
RFLP	Restriction Fragment Length Polymorphisms
RIN3	Ras and Rab interactor 3
RIP2	Receptor-interacting protein 2
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediate
ROC	Receiver operator characteristics
ROI	Reactive oxygen intermediates
sICAM	soluble intercellular adhesion molecule
SNP	Single nucleotide polymorphism
SOCS3	Suppressor of cytokine signaling 3
SpA	Surface protein A
ST	Shared type
SPP1	Secreted phosphoprotein 1
TB	Tuberculosis
TCR	T cell receptor
TEX264	Testis-expressed gene 264
TGF β	Tumour growth factor-β
TH 2	T helper 2
TH1	T helper 1
TH17	T helper 17
TIMP2	Tissue inhibitors of matrix metalloproteinase
TLR	Toll like receptor
TNF	Tumour necrosis factor
TNFRS	Tumour necrosis factor receptor superfamily
TRAF6	TNF receptor associated factor
VEGF	Vasicular epidermal growth factor
WHO	World Health Organization
αβ	Alpha beta
γδ	Gamma delta

Abstract

The immune response against M. tuberculosis is multifactorial, involving a network of innate and adaptive immune responses. Characterization of the immune response, a clear understanding of the dynamics and interplay of different arms of the immune response and the identification of infection-stage specific biomarkers are critical to allow the development of better tools for combating tuberculosis. In an attempt to identify such biomarkers, we studied pulmonary tuberculosis patients and their contacts in Addis Ababa, Ethiopia as part of EDCTP and BMGF funded tuberculosis projects by using multiplex techniques. We analysed 45 genes using the Multiplex Ligation Dependent Probe Amplification (MLPA) technique and the expression of IL-4d2, BLR1, MARCO, CCL-19, IL7R, Bcl2, FcgR1A, MMP9, and LTF genes discriminate TB cases from their healthy contacts. FoxP3, TGFb1 and CCL-19 discriminate latently infected from uninfected contacts. Single genes predict with an area under the Receiver Operating Characteristic (ROC) curve of 0.68 to 0.85 while a combination of genes identified up to 95% of the different groups. Similarly, the multiplex analysis of cytokines and chemokines also showed that single or combinations of plasma cytokines and chemokines discriminate between different clinical groups accurately. The median plasma level of EGF, fractalkine, IFN-γ, IL-4, MCP-3 and IP-10 is significantly different ($p<0.05$) in active tuberculosis and non active tuberculosis infection and the median plasma levels of IFN-γ, IL-4, MCP-3, MIP-1β and IP-10 were significantly different ($p<0.05$) before and after treatment. We also found a significant difference ($p<0.05$) in plasma levels of cytokines of patients infected with the different lineages and different families of the modern lineage. The plasma level of IL-4 was significantly higher in patients infected with lineage 3 ($p<0.05$) as compared to lineage 4 and the CAS family-infected patients had a higher plasma level of IL-4 ($P<0.05$) as compared to patients infected with H and T families but there was no difference between H and T families.

We identified genes and cytokines which had been reported from other studies in different settings and we believe that these molecules are very promising biomarkers for classifying active tuberculosis, latent infection, absence of infection and treated infection. These markers may be suitable for the development of clinically useful tools but require further validation and qualification in different populations and in larger studies.

Abstrak

Die immuunrespons teen M. tuberculosis is multifaktoriaal en betrek ‘n netwerk van nie-spesifieke and spesifieke immuunresponse. Karakterisering van die immuunrespons, ‘n duidelike insig in die dinamika en tussenspel deur die verskillende arms van die immuunrespons en die identifikasie van spesifieke biomerkers is krities belangrik om die ontwikkeling van nuwe hulpmiddels teen tuberkulose te bevorder. In ‘n poging om sulke biomerkers te identifiseer het ons pulmonale tuberkulose pasiënte en hulle kontakte in Addis Ababa, Etiopië, as deel van die EDCTP en BMGF befondste tuberkulose projekte bestudeer met multipleks tegnieke. Ons het 45 gene analiseer met ‘Multiplex Ligation Dependent Probe Amplification (MLPA)’ en gevind dat die geenuitdrukking van IL-4•2, BLR1, MARCO, CCL-19, IL7R, Bcl2, Fc•R1A, MMP9, en LTF TB pasiënte van hulle kontakte onderskei. FoxP3, TGF•1 en CCL-19 onderskei tussen latent infekteerde en ongeïnfekteerde kontakte. Enkele gene voorspel met ‘n area onder die ‘Receiver Operating Characteristic (ROC)’ kurwe van 0.68 tot 0.85 terwyl die kombinasie van gene 95% van die verskillende groepe identifiseer. Soortgelyk het multipleks analise van sitokiene en chemokiene verskillende kliniese groepe akkuraat van mekaar onderskei. Die mediane plasmavlakke van EGF, fractalkine, IFN-•, IL-4, MCP-3 en IP-10 is beduidend verskillend ($p<0.05$) in aktiewe tuberkulose en nie-aktiewe tuberkulose infeksie en die mediane plasmavlak van IFN-•, IL-4, MCP-3, MIP-1• en IP-10 was beduidend verskillend voor en na behandeling. Ons het ook beduidende verskille ($p<0.05$) in plasmavlakke van sitokiene in pasiënte gevind wat infekteer is met verskillende stamme and verskillende families van die moderne stamme. Die plasmavlak van IL-4 was beduidend hoër in pasiënte wat infekteer is met stam 3 ($p<0.05$) teenoor stam 4 en die CAS familie-infekteerde pasiënte het ‘n hoër plasmavlak van IL-4 ($p<0.05$) teenoor pasiënte met H en T familie infeksie hoewel daar geen versikke was tussen die H en T families nie.

Ons het gene en sitokiene identifiseer wat deur ander werkers onder verskillende omstandighede ook beskryf is en ons glo dat hierdie molekules baie belowende biomerkers is om aktiewe tuberkulose, latent tuberkulose, die afwesigheid van infeksie en behandelde infeksie van mekaar te onderskei. Hierdie merkers mag toepaslik wees vir die ontwikkeling van bruikbare kliniese hulpmiddele maar benodig verdere validasie en kwalifikasie in verskillende populasiegroepe en in groter studies.

CHAPTER 1

1. General Background

1.1 Introduction

1.1.1 Tuberculosis

Tuberculosis (TB) is one of the most devastating diseases of mankind and remains a major health threat in Africa with much higher rates in the Sub-Saharan part of the continent. The Mycobacterium tuberculosis complex (MTBC) is the cause of TB and encompasses M. tuberculosis, M. bovis, M. africanum, M. canettii, M. pinnipedii, M. caprae, and M. microti, which have 99% genetic similarity and identical 16S sRNA (Boddinghaus et al.; 1990; Sreevatsan et al.; 1997). M. tuberculosis is the predominant cause of TB in humans.

M. tuberculosis is an acid fast, facultative intracellular aerobic pathogen that has straight or curved rod morphology and exists either singly or in clusters. M. tuberculosis is a slow growing bacterium and divides once every 18-24 hours requiring 18-21 days before visible colonies develop on solid medium (Salyers and Whitt; 1994). The cellular envelope of M. tuberculosis consists of a plasma membrane and a highly unusual cell wall. The plasma membrane consists of a classical bilayer structure. The elaborate distinctive features of the mycobacterial cell walls include the lipoarabinomannan (LAM), lipomannan, mycolyl-arabinogalactan, phosphatidyl-myoinositol mannoside, sulfatide, cord factor, and other acylated trehaloses, phenolic glycolipids, lipoligosaccharides, and other attenuated lipids. Many of these have been shown to be involved in the virulence and pathogenesis of this bacillus. LAM, a predominant component of the cell wall, is a virulence factor for M. tuberculosis, which activates macrophages (MØ) and scavenges reactive oxygen intermediates (Brennan, PJ and Nicaido, H ; 1995; Nigou et al.; 2003).

1.1.2 Epidemiology of Mycobacterium tuberculosis infection

Mycobacterium tuberculosis was first identified as the causative agent of TB by Robert Koch in 1882. It is an extraordinary effective human pathogen infecting one-third of the world's population with only 10% of these people developing active disease from the primary infection, in most cases within the first two years, whereas the remaining 90% of cases remains non-infectious and symptom free. The presence of underlying factors, which weaken the immune system, including chronic diseases like diabetes, alcoholism, malnutrition, stress and above all HIV/AIDS, increase the risk of developing the disease. M. tuberculosis infected HIV positive subjects have a 10% annual risk of developing active TB as opposed to the 10% life time risk in HIV negative individuals (Kaufmann and McMichael; 2005).

TB is the world's second most common cause of death from all infectious diseases, next to HIV/AIDS. In 1993 the World Health Organization (WHO) declared TB as a 'global emergency' (WHO; 1994). There were 8.8 million new cases of TB in 2010, 1.1 million (range, 0.9–1.2 million) deaths from TB among HIV-negative people and an additional 0.35 million (range, 0.32–0.39 million) deaths from HIV-associated TB. Of the 8.8 million incident cases in 2010, 1.1 million (13%) were among people living with HIV. Asia and Africa reported most numbers of cases in 2010 with 59% and 26% respectively; smaller proportions of cases occurred in the Eastern Mediterranean Region (7%), the European Region (5%) and the Region of the Americas (3%) (Figure 1-1). The 22 High Burden Countries (HBCs) accounted for 81% of all estimated cases worldwide (WHO; 2011).

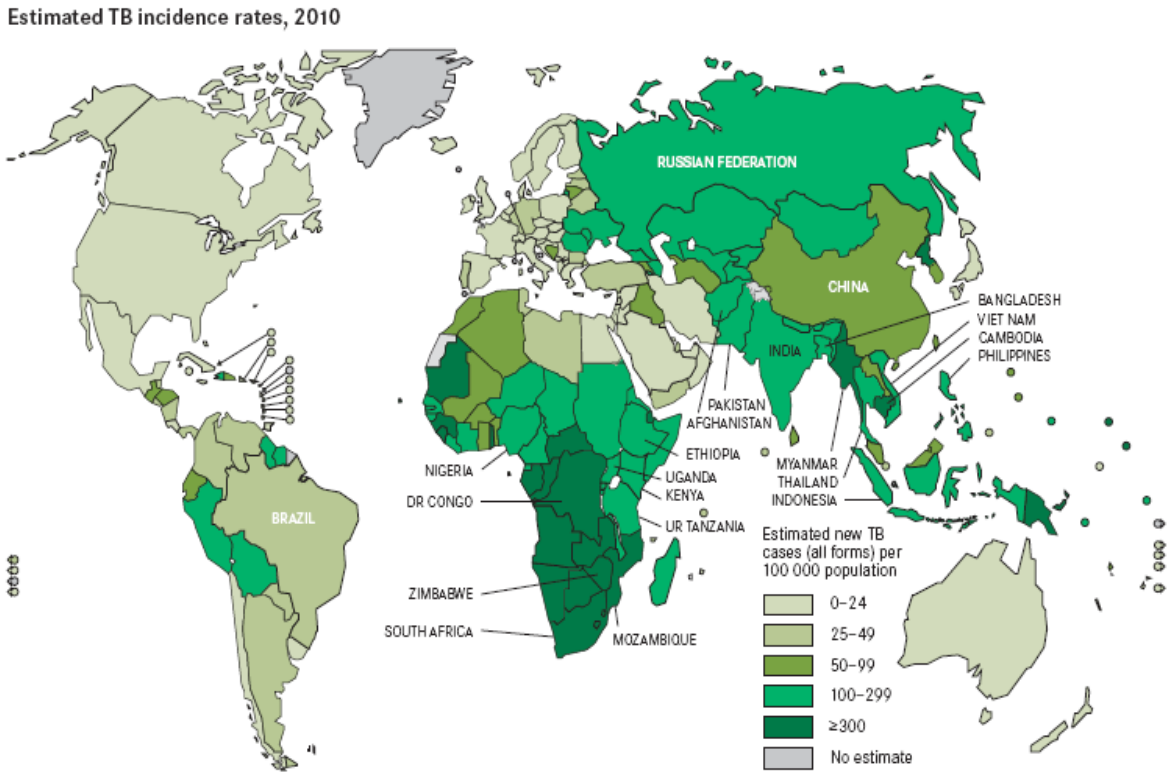
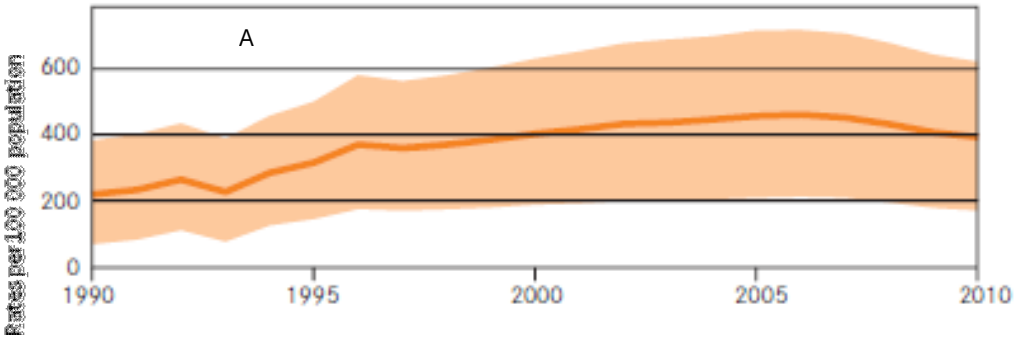


Figure 1-1 Global Tuberculosis incidence rates, 2010 (WHO; 2011) .
The 22 high burden countries are listed by name and nine of them are in Sub-Saharan Africa

In Ethiopia, the incidence of TB is estimated at 240 new cases per 100,000 populations with a prevalence of 394 per 100,000 populations and the country is rated 7th among the 22 high burden countries. The mortality rate of all cases of TB is estimated to be 35 per 100,000 populations (figure 1-2). Fifteen percent of tested TB patients are HIV positive (WHO; 2011).



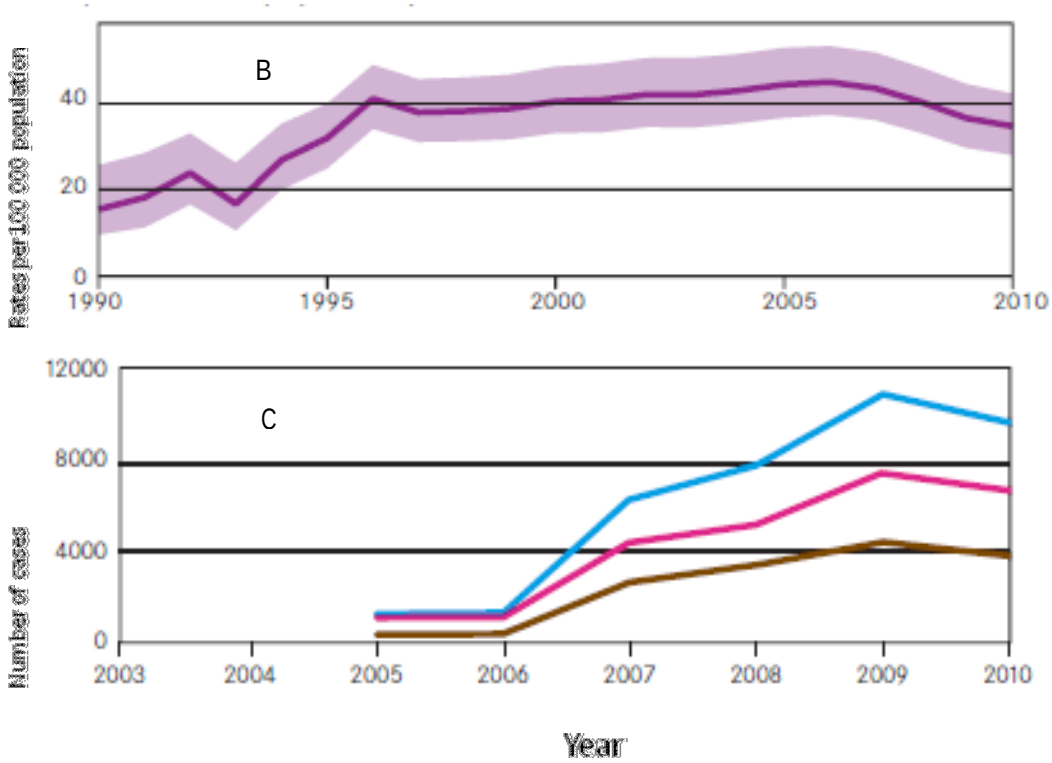


Figure 1-2 Tuberculosis trends in in Ethiopia (WHO; 2011).

Trends in estimated prevalence of TB in Ethiopia 1990-2010 (A), mortality excluding HIV 1990-2010 (B) and HIV-TB positive patients (blue) who are taking co-trimoxazole preventive therapy (CPT) (pink) and ART (brown) 2005-2010 (C). The shading areas in"A"and "B" represent uncertainity areas

1.1.3 Pathogenesis of tuberculosis

The inhalation of small size respiratory droplet nuclei (1-2µm or less) through the respiratory tract is the commonest route of entry of the tubercle bacillus. The respiratory

droplet nuclei are small enough in size to pass into the lower respiratory tract escaping the anatomical barriers of nasopharynx and upper respiratory tract (Schluger and Rom; 1998). M. tuberculosis does not infect the respiratory bronchial epithelium (McDonough and Kress; 1995) and studies indicated that the bronchial epithelium can produce antimicrobial peptides with a wide spectrum of activity (Diamond et al.; 1991). Phagocytic cells mainly macrophages take up the bacteria once inhaled droplets pass into the lower respiratory

tract and are deposited in the alveolar spaces, which assist in the induction of a rapid inflammatory response and accumulation of cells. Although alveolar macrophages are the first cells to engulf, dendritic cells and monocyte-derived macrophages also take part in the phagocytic process (Henderson et al.; 1997; Thurnher et al.; 1997).

Endocytosis of M. tuberculosis involves multiple receptors (Figure 1-3) such as complement, FcR, surfactant protein A (Sp-A) and its receptors, scavenger receptor class A, TLR, CD14 mannose receptors and the DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) (Ernst; 1998). Some receptors allow silent entry (CR), and others induce defense mechanisms (FcR) (Hirsch et al.; 1994; Aderem and Underhill; 1999). The subsequent intracellular fate of mycobacteria is considered as predetermined by the mode of entry into macrophages (Kleinnijenhuis et al.; 2011), however, experiments have shown that intracellular trafficking of M. tuberculosis was not significantly altered by blocking individual receptors (Ernst; 1998).

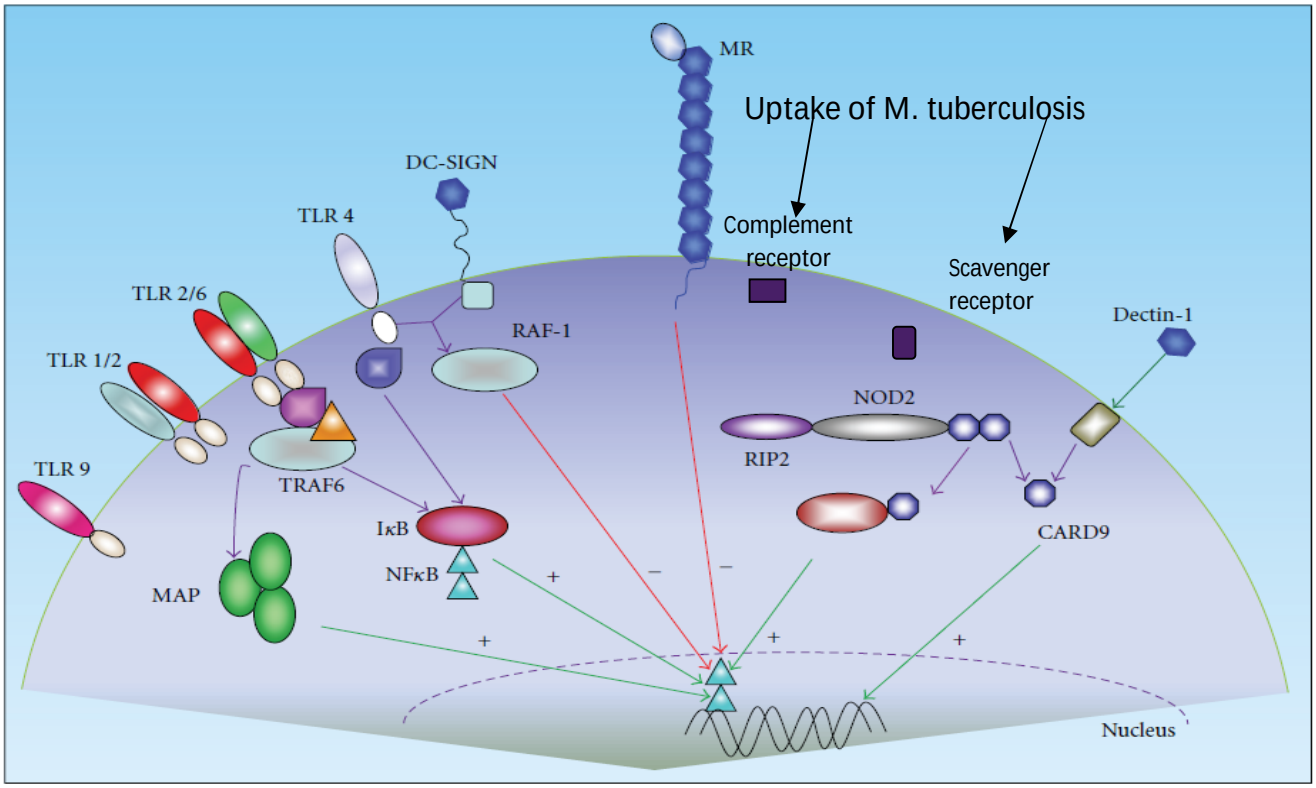


Figure 1-3 Endocytosis receptors in the recognition of mycobacteria (Kleinnijenhuis et al.; 2011).
Both intracellular and extracellular receptors are involved in this process. Complement receptors are primarily responsible for uptake of opsonized M. tuberculosis and scavenger receptors for uptake of nonopsonized M. tuberculosis. TLRs play a central role in immune recognition of M. tuberculosis. Depending on the type of receptors, recognition of mycobacteria leads for intracellular cascading which in turn will lead to the activation of transcription of NF-•B, and induction of secretion of the of pro (+) and anti (-) inflammatory cytokines and chemokines.

Once organisms have made their way into the lung, they have four potential fates (van Crevel et al.; 2002; van Crevel et al.; 2003).

i. Killing and elimination of the bacilli with the initial host immune response, and these individuals do not develop TB due to this exposure event. No clinical or immunological evidence of this interaction is apparent.

ii. Immediately after infection the bacilli can grow and multiply, causing clinical disease (primary TB).

iii. Development of 'latent infection' where the bacilli persist in a sub clinical (quiescent) form. The bacteria may become dormant or may persist at low numbers, and are prevented from unchecked replication by the immune system and never cause disease at all. This phase is manifested only as positive tuberculin skin test (latent TB) or positive interferon gamma release assay.

iv. Reactivation of the dormant bacilli or escape from the quiescent phase with resultant disease (reactivation TB).

1.1.4 Immunology of tuberculosis

The protective response to M. tuberculosis is complex and multifaceted involving many components of the immune system, mainly the result of productive cooperation between macrophages and T-cell populations (Kaufmann et al.; 2005) (Figure 1-4).

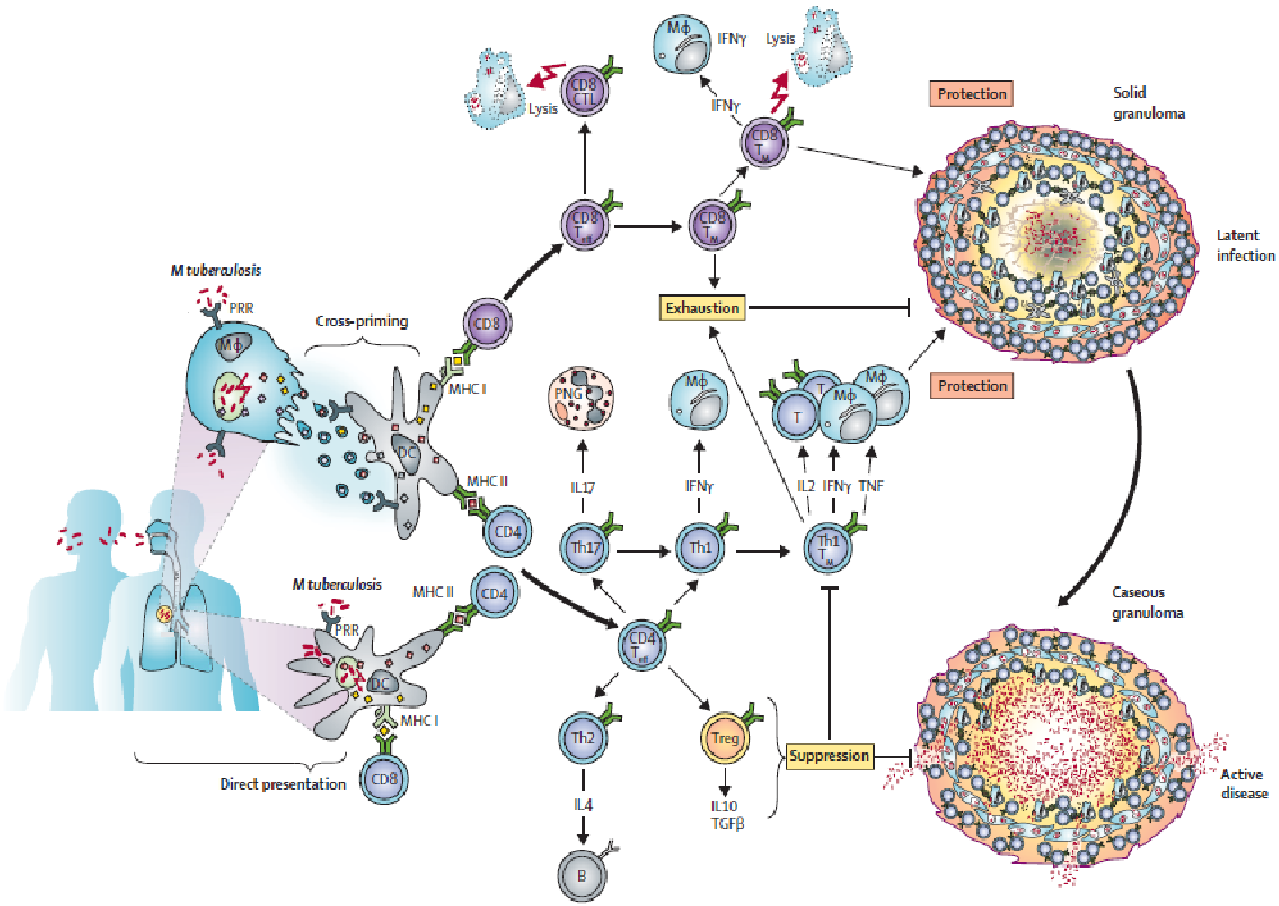


Figure 1-4 Immune responses to Tuberculosis (Kaufmann; 2010).

T cells and Macrophages are key cells in controlling M. tuberculosis, which survives inside the phagosomal compartment in macrophages and denritic cells. Exogenous mycobacterial peptides are presented to CD4 T cells in the context of MHC class II whereas mycobacterial antigens translocated into the cytosol, or cross-primed when macrophages release apoptotic bodies carrying mycobacterial peptides, are loaded on MHC I and presented to CD8 T-cell. Th1 cells produce IL-2 for T-cell activation as well as IFN-γ and TNF for macrophage activation. Th17 cells, which activate mainly neutrophils, contribute to the early formation of protective immunity in the lung after vaccination. Th2 cells produce IL-4 and regulatory T cells (Treg) TGF or IL-10 and counter-regulate Th1-mediated effects. CD8 T cells produce IFN–γ and TNF, which activate macrophages and also secret perforin and granulysin which lyses target cells and attack M. tuberculosis.

1.1.4.1 Macrophages

Macrophages are key cells in the immune response to M. tuberculosis by presenting antigens to T cells in the context of both MHC class I and II. The activated T cells in turn activate macrophages by secreting IFN-γ to kill the bacteria (Flynn and Chan; 2001). Activated macrophages also secrete an important proinflammatory cytokine, TNF, which induces antimycobacterial effect in synergy with IFN-γ (Bekker et al.; 2001). Furthermore, macrophages play an important role in recruitment of cells at the site of infection by secreting the proinflammatory cytokines IL-1 and IL-6 (Giacomini et al.; 2001).

Activated macrophages kill engulfed pathogenic bacteria via different mechanisms, including phagosome-lysosome fusion, generation of reactive nitrogen intermediates, particularly nitric oxide and generation of reactive oxygen intermediates. The maturation of phagosomes is a dynamic process where the phagosomes, which contain engulfed microbes fuse with lysosomes (Desjardins et al.; 1994; Desjardins; 1995). The phagolysosome fusion represents a major antimicrobial mechanism where engulfed microbes are degraded by intralysosomal acidic hydrolases (Flynn and Chan; 2001).

M. tuberculosis prevents phagolysosomal fusion and survives inside macrophages (Armstrong and Hart; 1971; Hart et al.; 1972). However, how mycobacteria modulate the phagosomal membrane to block further maturation into phagolysosomes remains largely unknown. It has been reported that mycobacterial sulfatides, derivatives of multiacylated trehalose 2-sulfate, a lysosomotropic polyanionic glycolipid and ammonia have the ability to inhibit phagolysosomal fusion (Flynn and Chan; 2001).

Reactive Nitrogen Intermediates

Macrophages have been shown to produce nitric oxide (NO) and other reactive nitrogen intermediates (RNI) via the NOS2 enzyme using L-arginine as a substrate. NOS2 is induced by IFN-γ and a second signal such as TNF-α or bacterial products such as LPS or LAM (MacMicking et al.; 1997). Nitric oxide production within macrophages has major anti-microbial mechanisms. RNI can inflict damage to the bacterium by modifying DNA, proteins and lipids. In murine models, toxic nitrogen compounds have been shown to play a role in protection in both an acute and a chronic M. tuberculosis infection (Shiloh; 2000).

Reactive Oxygen Intermediates

Unlike the role of NOS2, the importance of toxic oxygen species in the control of TB is not fully understood. Although ROI were shown to kill some species of mycobacteria like M. microti and H_2O_2 generated by cytokine activated macrophages was mycobacteriocidal, the effect of ROI on M. tuberculosis remains to be confirmed (Walker and Lowrie; 1981; Flesch and Kaufmann; 1987; Chan et al.; 1992). The mycobacterium cell wall component, LAM, scavenges and enables mycobacteria to escape the toxic effect of ROI (Chan et al.; 1989; Chan et al.; 1991). Tubercle bacilli also produce both superoxide dismutase and catalase that may interfere with toxic oxygen radical production.

1.1.4.2 Dendritic cells

Dendritic Cells (DC) are superior antigen presenting cells (APCs) and they help in maximizing the recognition of antigens by T cells in the draining lymph nodes (Gonzalez-Juarrero et al.; 2003; Flynn; 2004; Marino et al.; 2004). DC-SIGN is a receptor through which dendritic cells interact with microbes and its interaction with components of mycobacteria has been reported as one of the major examples how M. tuberculosis influence DC function (Tailleux et al.; 2003). The initiation of the immune response to limit infection during primary TB

occurs when immature DC capture M. tuberculosis, which leads to matured DC that migrate into draining lymphnode, present and stimulate T cells (Sertl et al.; 1986). In this compartment, T cells are activated, differentiate into effector and memory T cells and induce protection against M. tuberculosis in the lungs (Kaufmann; 2001; Kaufmann and Schaible; 2003).

1.1.4.3 T cells

M. tuberculosis is a classic example of a pathogen which resides intracellularly within macrophages and for which the protective response relies on cell-mediated immunity. Studies demonstrated that acquired immunity to M. tuberculosis requires contributions by multiple T cell subsets: CD4 T cells, CD8 αβ and γδ T cells and CD1 restricted T cells (Boom et al.; 2003).

1.1.4.4 CD4 T Cells

CD4 T cells play a dominant role in the protective response against M. tuberculosis (Kaufmann et al.; 2005). The mycobacteria reside within macrophage vacuoles; therefore, mycobacterial antigens are loaded on MHC class II and presented to CD4 T cells through endocytic antigen presentation pathway. Upon activation, CD4 T cells secret IFN-•, IL 2 and TNF, which in turn activates macrophages (Flynn and Chan; 2001). CD4 depleted or disrupted mice and adoptive transfer of CD4 cells showed that this population is important for controlling infections (Orme and Collins; 1983; Orme and Collins; 1984; Muller et al.; 1987; Tascon et al.; 1998; Caruso et al.; 1999). In humans, the necessity for CD4 T cells to help control of infection is shown by the rapid acceleration of TB in HIV positive patients who have loss of CD4 T cells (Selwyn PA; 1989). Moreover, CD4 cells are also important to enhance APC function through interaction of CD40-CD40L between CD4 T cells and APC and

this in turn can facilitate APC medicated induction of other T cells such as CD8 T cells (Andreasen et al.; 2000).

Studies in CD4 depleted or deficient mice have also shown that CD4 cells have other roles in addition to IFN-γ production in controlling mycobacterial infections (Scanga et al.; 2000). CD4 T cells induce apoptosis through perforin and granulysin, FAS-L or TNF-α lytic pathways (Oddo et al.; 1998), provide help for B cells and CD8 T cells, and produce other cytokines (Keane et al.; 1997), including those that limit immunopathology.

1.1.4.5 CD8 T Cells

The MHC class I-restricted CD8 T cells contribute to protective immunity against TB (Kaufmann; 2006; Cooper; 2009) however, mechanisms underlying CD8 T cell stimulation are not fully understood. The stimulation of CD8 T cells requires mycobacterial peptide presentation by MHC I products, which generally occurs in the cytosol which is not readily accessed by M. tuberculosis. Two pathways have been proposed: first, M. tuberculosis can enter into the cytosol of infected DCs, and this leads to direct loading of MHC I molecules (van der Wel et al.; 2007). Secondly, apoptosis of macrophages infected with M. tuberculosis results in mycobacterial antigen carrying vesicles, which are taken up by DCs and leads to cross priming (Winau et al.; 2006). CD8 T cells that are specific for mycobacterial antigens can produce IFN-γ and secrete perforin and granulysin, which lyse host cells and attack M. tuberculosis directly (Stenger et al.; 1997).

1.1.4.6 Unconventional T cells

CD1 restricted CD4 and CD8 T cells and γδ T cells participate in the response against M. tuberculosis in humans (Kaufmann; 1996; Lewinsohn et al.; 1998; Lewinsohn et al.; 2000). CD1 molecules present glycolipid antigens, which are found in the cell wall of mycobacteria.

These cells secrete IFN-γ and express cytolytic activity (Porcelli and Modlin; 1999; Ulrichs and Porcelli; 2000).

Studies in mice showed that γδ T cells partially protect against high doses of M. tuberculosis infection and they are important in regulating granuloma formation (Ladel et al.; 1995; D'Souza et al.; 1997). In humans, these cells comprise about 5% of the whole T cell population in peripheral blood (Kaufmann; 1996). The γδ cells have a mycobacteriocidal activity via the release of their granules and stimulation of these cells with phospholigands also induces IFN-γ production (Behr-Perst et al.; 1999). Therefore, these cells are believed to be part of the first line of defense against TB.

1.1.5 Cytokines

Cytokines are proteins, which play a role in disease protection, progression or development of pathophysiology. Different animal and human studies have firmly established that cytokines have a major role in determining the outcome of infection with M. tuberculosis.

Interferon-g (IFN g)

Conventional CD4 and CD8 T cells are considered as the primary source of IFN-γ and from mouse and human studies it is a well-established fact that IFN-γ is a critical cytokine involved in the control of M. tuberculosis infection. Mice with a genetic deficiency of IFN-γ are very susceptible to infection with virulent M. tuberculosis with a shorter mean survival time and less NOS2 production, indicating that macrophage activation was defective, contributing to the susceptibility of IFN-γ gene knockout (KO) mice (Flynn et al.; 1993). The importance of this cytokine has also been confirmed in humans who have a mutation in

their IFN-γ receptor genes, who display heightened susceptibility to mycobacterial infections (Jouanguy; 1999).

IFN-γ can also be produced by γδ T cells, NKT cells and NK cells and both in vitro and in vivo studies showed that these cells can display protective effects against M. tuberculosis. However, the role of these cells in the presence of adaptive T cells is not clear and it is believed that these cells may serve as secondary sources of IFN-γ at time of heavy or hypervirulent mycobacterial exposure (Cooper et al.; 2011).

Tumour Necrosis Factor a (TNF-a)

Tumor necrosis factor plays a central role in the initiation and maintenance of controlling M. tuberculosis by activating macrophages and facilitating granuloma formation (Flynn et al.; 1995a; Schaible et al.; 1999; Dinarello; 2003). TNF-α, in synergy with IFN-γ, activates macrophages to produce RNI and mice deficient in TNF-α or the 55-kDa TNF receptor succumbed to infection (Flynn et al.; 1995a; Bean et al.; 1999). TNF-α or its receptor also affects cell migration and the granulomatous response following M. tuberculosis infection (Vaday et al.; 2001). In addition, reactivation of latent disease in rheumatoid arthritis patients after neutralization of TNF with specific monoclonal antibodies signifies the importance of this cytokine (Keane; 2005).

Interleukin 12 (IL-12)

A type 1 T cell response must be generated to control M. tuberculosis infection as is the case for most intracellular infections. The susceptibility of IL-12p40 gene deficient mice to M. tuberculosis and the therapeutic role of IL-12 strongly support an important role for this

cytokine in the protective immune response against M. tuberculosis (Flynn et al.; 1995b; Cooper et al.; 1997). In chronically M. tuberculosis infected mice administration of IL-12 DNA has been reported to reduce the bacterial load (Lowrie; 1999). Mutations in the IL-12p40 receptor IL-12RB1 gene are also strongly associated with susceptibility to TB (Altare et al.; 1998).

Interleukin 10 (IL-10)

IL-10 is an anti-inflammatory cytokine, possesses macrophage deactivating properties which in turn leads to down regulation of IL-12 and TNF and consequently IFN-γ secretion by T cell and MØ activation (Turner et al.; 2002; Beamer et al.; 2008). Suppression of T cell proliferation in vitro by MØs isolated from human TB patients could be reversed by inhibition of IL-10 (Gong J-H; 1996). CD4 T cell responses and APC functions during mycobacterium infection has been shown to be directly inhibited by IL-10 (Rojas et al.; 1999).

Tumour Growth Factor b (TGF-b**)**

TGF-β is a classic anti-inflammatory cytokine, which counteracts protective immunity against tuberculosis by inhibiting T cell proliferation and IFN-γ production. It antagonizes antigen presentation, proinflammatory cytokine production, and cellular activation in macrophages by inhibiting IFN-γ induced NOS2 production (Ding et al.; 1990; Hirsch et al.; 1997; Rojas et al.; 1999). During TB TGF-β is produced in excess and is expressed at the site of disease (Toossi; 1995).

Interleukin 4 (IL-4)

The role of IL-4 in TB is subject of some controversy. Increased production of IL-4 in mice infected with M. tuberculosis has been associated with progressive disease (Hernandez Pando and Larriva Sahd; 1996) and reactivation of latent infection (Howard; 1999) as IL-4 suppresses IFN-γ production, and macrophage activation (Lucey; 1996). Conversely, IL-4 KO mice displayed normal instead of decreased susceptibility to mycobacteria (North; 1998).

Interleukin 17 (IL-17)

IL-17 is a relatively newly identified cytokine and it is important for recruiting neutrophils and repairing tissues and its role in the immune response against rapidly growing extracellular pathogens has been shown in different studies (Ye et al.; 2001a; Ye et al.; 2001b; Happel et al.; 2005). The γδ T cell population is a major source of early IL-17 during mycobacterial infection (Lockhart et al.; 2006; Umemura et al.; 2007). After low dose aerosol infection, the ability of mice to control M. tuberculosis is not significantly affected by the absence of IL-23, a DC derived inducer of IL-17 or IL-17 signaling (Khader et al.; 2005; Aujla et al.; 2007). However, mice deficient in IL-17 were unable to control M. tuberculosis after high dose intratracheal infection (Okamoto Yoshida et al.; 2010). IL-17 is mainly associated with its crucial role in early granuloma formation and efficient mycobacterial killing via neutrophil recruitment and triggering proinflammatory programs associated with chemokine secretion (Seiler et al.; 2003; Silva; 2010).

1.1.6 Chemokines

Chemokines (chemotactic cytokines) are largely responsible for cell trafficking to the site of infection, however, their role in M. tuberculosis infection have been investigated to a limited extent. M. tuberculosis induces elevated levels of a variety of chemokines, including IL-8 (CXCL-8), monocyte chemoattractant protein 1 (MCP-1) (CCL-2), MCP-3 (CCL-7), MCP-5

(CCL-12), Regulated on Activation Normal T Cell Expressed and Secreted (RANTES/CCL-5), Macrophage Inflammatory protein (MIP1)-α (CCL-3), MIP1-β (CCL-4), MIP-2 (CXCL-2), and IP-10 (CXCL-10) (Rhoades et al.; 1995; Orme and Cooper; 1999) and their receptor like CCR5 and CXCR4 (Juffermans et al.; 2000).

1.2 Mycobacterium tuberculosis genetic diversity and its effect on immune responses

The sequencing of the complete genome of M tuberculosis in 1998 helps significantly in developing different genotyping methods to characterize strains according to their genome differences. Different molecular techniques have been used to characterize strain to strain variation in M. tuberculosis and studying molecular epidemiology of M. tuberculosis in different population assists in understanding their global distribution. The genome of M. tuberculosis is highly conserved compared with other pathogenic bacteria and it exhibits no or very limited horizontal gene transfer. However, the presence of Single Nucleotide Polymorphism (SNP), deletions, insertions and/or repetitive elements has led to many polymorphisms in the M. tuberculosis genome. M. tuberculosis isolates have been characterized to strain level by using different genetic markers which are specific in different clinical isolates (Bifani et al.; 2002). The genetic biomarkers include Spacer Oligonucleotide Types (spoligotypes), Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeats (MIRU-VNTRs), IS6110 Restriction Fragment Length Polymorphisms (RFLP), Long Sequence Polymorphisms (LSPs) and Single Nucleotide Polymorphisms (SNPs) (Shabbeer et al.; 2011).

The global population structure of M. tuberculosis is defined by six phylogeographical lineages based on LSP: Indo-Oceanic lineage, East Asian lineage, East African-Indian lineage,

Euro-American lineage, West African lineage I and West African lineage II (Gagneux and Small; 2007). The Indo-Oceanic lineage (Lineage I), West African lineage I (Lineage 5) and West African lineage II (Lineage 6) are belonging to ancient lineages whereas the East Asian lineage (Lineage 2), East African-Indian lineage (Lineage 3) and Euro-American lineage (Lineage 4) belong to the modern lieage (Gagneux et al.; 2006). The lineage names reflect particular geographical areas where each lineage is found most commonly and geographically structured (Figure 1-5). For example, India is dominated by the Indo-Oceanic lineage, the Far East by the East-Asian lineage and the Euro-American lineage is the dominant lineage in Europe and the Americas. In contrary to the other regions where a single lineage is more dominant, all six main lineages are represented in Africa. These lineages included both ancient and modern lineages (Gagneux et al.; 2006).

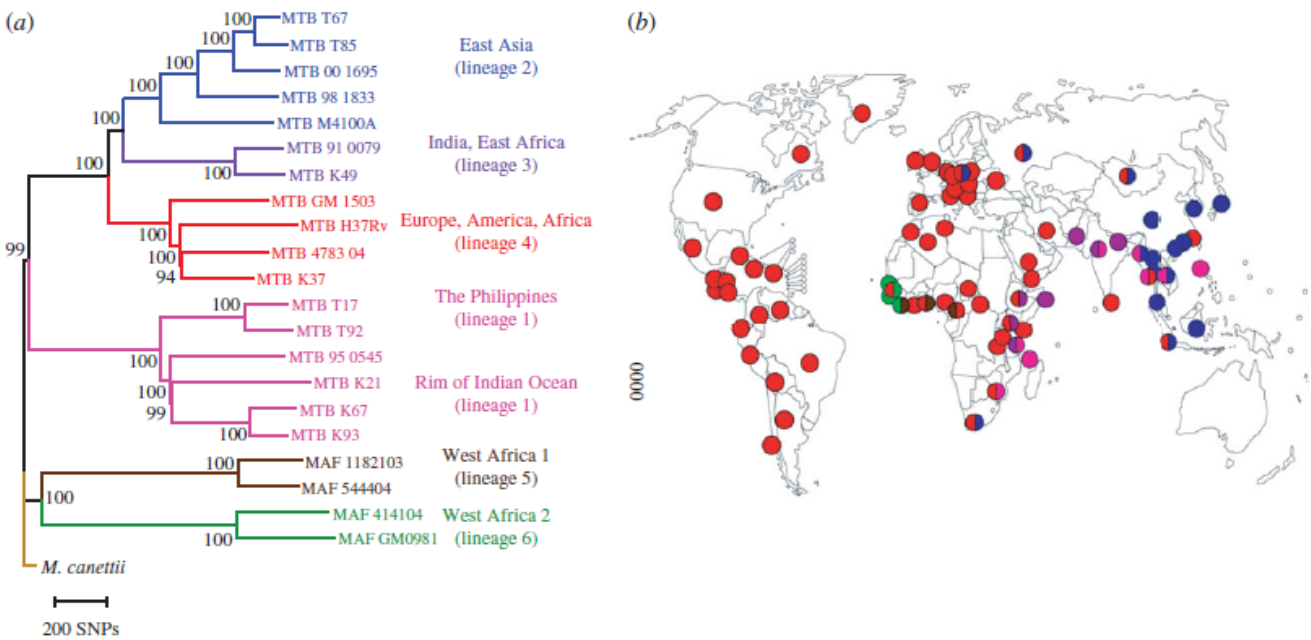


Figure 1-5 Global phylogeography of MTBC (Gagneux; 2012).
(a) Phylogeny of the six MTBC lineages (b) Distribution of the six MTBC lineages. Colored dots represent the major lineages in the respective countries

The environmental and host factors are important elements affecting the outcome of infection and disease presentation (Bellamy; 2005; Lonnroth et al.; 2009), however, the role of bacterial factors particularly strain-to-strain variation is not clearly understood. The existing dogma of rare allelic polymorphism in this organism compared with other bacteria, which leads to low levels of genetic variation in M. tuberculosis, has lead to the assumption that strain diversity would have no clinical significance. However, the application of the different genotyping methods and recent advances in mycobacterial genomics and population genetics show that the genomic variation in M. tuberculosis might have been underestimated, and that the different phenotypic characteristics of the pathogen may be associated with its genotype (Nicol and Wilkinson; 2008).

Studies suggest that lineages differ in their natural history of infection, presentation of disease, immune response and response to treatment (Gagneux et al.; 2006; Caws et al.; 2008; Thwaites et al.; 2008; Burman et al.; 2009; van der Spuy et al.; 2009). Gagneux and colleagues, recently showed that specific relationships between the genome of the host and genotype of the pathogen affects the ability of different lineages of M. tuberculosis to cause secondary cases (Gagneux et al.; 2006). Nahid and colleagues also showed that cavitary disease in participants from the African region caused by East Asian lineage isolates may be less responsive to combination therapy (Nahid et al.; 2010). Similarly, another study in South Africa reported a higher likelihood of treatment failure in patients infected by members of the Beijing family (van der Spuy et al.; 2009). Other studies reported an association between disease progression in tuberculosis meningitis and the East Asian lineage. The Euro-American lineage causes a limited extent of tuberculosis disease and are less able to cause disseminated or meningeal tuberculosis (Caws et al.; 2008; Thwaites et al.; 2008).

Beijing M. tuberculosis strains have shown marked virulence in animal models of infection (Mathema et al.; 2006) and it has been suggested that certain subgenotypes of Beijing may have increased transmissibility and/or pathogenicity (Hanekom et al.; 2007). Many reports from Germany, Italy, Russia, Estonia, South Africa and Columbia documented that the isolates identified as Beijing genotype were associated with multiple drug resistance (Caminero et al.; 2001; Drobniewski et al.; 2002; Filliol et al.; 2002; Lari et al.; 2004; Cowley et al.; 2008). Other studies have found Beijing strains to be associated with HIV (Middelkoop et al.; 2009).

M. tuberculosis interferes with the host immune system in different ways (Flynn and Chan; 2005). Several studies suggested that the genetic diversity of M. tuberculosis affects some characters of the pathogen where one genotype induces more proinflammatory cytokines whereas the other induces a higher level of anti-inflammatory cytokines. For example, Strain CDC1551 and Strain HN878 differ in virulence and this has been linked to their cell wall lipid components. Studies indicate that cytokines which are characteristic of a protective immune response including TNF-•, IL-1, IL-12, and IFN-γ were upregulated in human monocytes and mice by CDC1551 or its lipid extracts (Manca et al.; 1999; Manca et al.; 2004).

On the other hand, increased production of macrophage deactivating cytokines such as IL-11 and IL-13 and reduced expression of proinflammatory cytokines such as IL-6, TNF and IL-12 has been associated with strain HN878 due to the production of a phenolic glycolipid (PGL). Production of type 1 interferon (α and β), which has been associated with decreased survival in mice, was observed in infection with strain HN878 (Manca et al.; 2001; Manca et al.; 2004).

Macrophages infected with strain CH of the East African-Indian lineage expressed and secreted less protective IL-12p40 and more regulatory IL-10. A high proportion (23%) of subjects infected with this strain had progressed to active disease within a year and the deletion of Rv1519 in this strain was taken as a reason why the innate immunity was diminished (Newton et al.; 2006). Another study conducted in Madagascar indicated that new or modern strains induced a host response different from that of ancient strains. In this longitudinal study they reported a lower IFN γ response in TB cases infected with modern M. tuberculosis strains, like Beijing and Central Asian (CAS) strains than those infected with ancient strains like East African-Indian (EAI) strains. They also found a similar result in household contacts according to the genotype of the strains isolated from their respective index cases (Rakotosamimanana et al.; 2010). A study in The Gambia also showed an attenuated T-cell response to early secreted antigenic target 6 (ESAT6) in TB patients and their household contacts infected with West African lineage II (Rakotosamimanana et al.; 2010).

1.3 Biomarkers of tuberculosis

The vigorous applications of the existing strategies and the coordinated efforts in the past years to control TB have had a positive effect on disease incidence, but the elimination of the disease is a log way off. Lack of effective vaccines, drugs, unavailability of simple diagnostic methods, spread of HIV/AIDS in TB-endemic regions and emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR-TB) TB strains, makes all these efforts unfruitful.

This experience has led to more research on innovating simple and rapid diagnostic tests, new drugs and vaccines. The current widely used diagnostic technique, therapeutic drugs

and vaccines were licensed a very long time ago and are inefficient in detecting, treating or protecting against tuberculosis. The lack of reliable simple biomarkers to indicate or predict the different clinical outcomes of M. tuberculosis infection has been given as a key reason for the failure of developing new tools, drugs and vaccines against tuberculosis.

A biological marker, or biomarker, is a characteristic that is objectively measured and evaluated as an indicator of a normal physiological or pathological process or pharmacological response(s) to a therapeutic intervention (Group; 2001). Host or pathogen specific TB biomarkers provide prognostic information, either for individual patients or study cohorts, about future health status and can advance knowledge of disease pathogenesis in predicting reactivation and cure, and indicating vaccine-induced protection (Figure 1-6). Biomarker studies use urine, saliva, breath and sputum samples to identify molecules for indicating or predicting the different clinical outcome of M. tuberculosis infection, however, peripheral blood remains an attractive sample type due to the ease with which this sample can be obtained. Gene transcripts, proteins, lipids and metabolites can all be measured in blood for biomarker studies (Parida and Kaufmann; 2010).

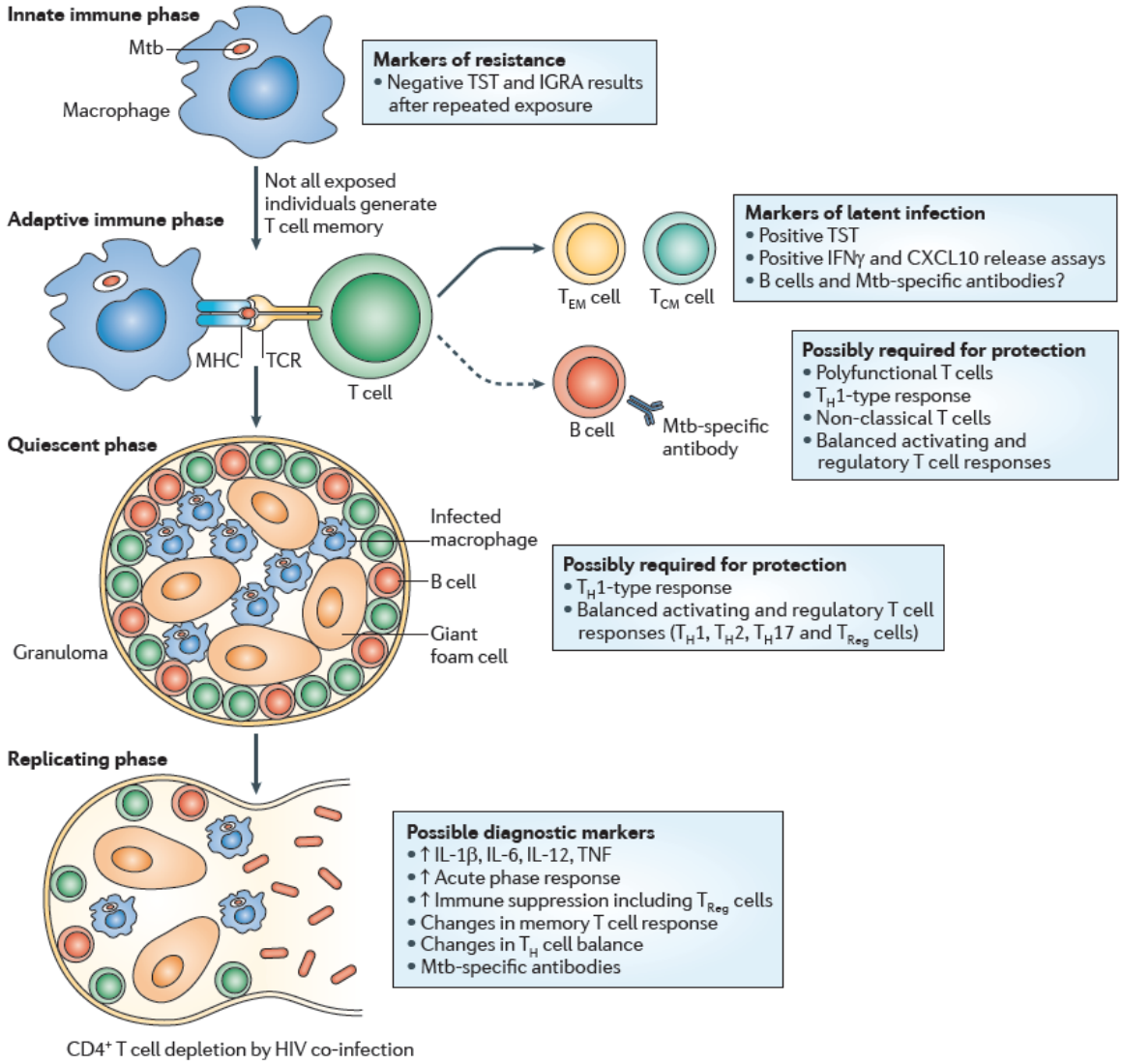


Figure 1-6 Host Biomarkers of Tuberculosis (Walzl et al.; 2011).
The different clinical outcomes of M. tuberculosis infection are determined by the different cells and molecules of both the innate and adaptive immune system. Infection with M. tuberculosis has different outcomes, which includes absence of any clinical or laboratory evidence of infection, latent infection and active disease. Application of a single or combination of markers could discriminate the different clinical groups.

The century old sputum smear microscopy is the most widely used test in high endemic countries and this test is inadequate to diagnose early active TB disease and unable to diagnose extra-pulmonary TB, sputum smear-negative TB (active pulmonary TB with less than 10,000 bacilli per ml of sputum) and childhood TB. Studies have indicated that Interferon Gamma Release Assays (IGRAs) show stronger responses in people with active TB than in those with latent TB (Janssens; 2007). Recent studies reported that IL-2, IFN-γ (Casey et al.; 2010) and TNF-α expression profiles of CD4 T cells (Harari et al.; 2011) hold promise in detecting active TB disease. Polycytokine signatures including EGF, sCD40L, MIP-1•, VEGF, TGF-• or IL-1• were reported to differentiate active TB disease from latent TB (Chegou et al.; 2009). In addition, the RNA expression level of CXCL-8, FoxP3 and IL-12• differentiates latent TB infection from disease (Wu et al.; 2007). Detection of circulating antibodies for diagnostic of prognostic potential are suggested in studies indicating correlation of M. tuberculosis-specific antibodies with bacterial burden (Kunnath-Velayudhan et al.; 2010). Combined expression patterns of Fc gamma receptor 1B (FcγR1B), FcγR1A (CD64), RAB33A and lactoferrin (LTF) (Jacobsen et al.; 2007; Maertzdorf et al.; 2011) and RIN3, LY6G6D, TEX264, and C14orf2 (Mistry et al.; 2007) genes also showed a discriminating power between active TB and LTBI.

Sputum culture or smear microscopy status after 2 months of therapy has been used as a surrogate marker for prediction of non-relapsing cure. The increased level of M. tuberculosis antigen 85 and 85B RNA in sputum protein during the first week of treatment predicted relapse or failure (Wallis et al.; 1998). Levels of IFN-γ (Chee et al.; 2010), IL-10, the ratio of IFN- γ/IL-10 (Sai Priya et al.; 2010) and IL-4/IL-4 • 2 (Wassie et al.; 2008) have been reported to have an association with treatment outcome. There are also other molecules which

decrease after treatment, like soluble intercellular adhesion molecule (sICAM)-1 (Lai et al.; 1993; Mukae et al.; 2003; Walzl et al.; 2008), C-reactive protein (Plit et al.; 1998) soluble urokinase plasminogen activator receptor (Eugen-Olsen et al.; 2002) and procalcitonin (Baylan et al.; 2006; Prat et al.; 2006).

High or increasing concentrations of TB specific IFN-γ production might predict overt TB (Doherty et al.; 2002; Higuchi et al.; 2008). The relative mRNA levels of IFN-γ, IL-4, and IL-4 • 2 have been reported as a better marker than IFN-γ alone, since ratios of IFN-γ to IL-4 and IL-4 • 2 to IL 4 decreased when contacts developed TB and increased in cured TB cases (Siawaya et al.; 2008; Wassie et al.; 2008). In chronic or long term latently infected individuals, the ration of IL-4 to IL-4 • 2 is also decreased, probably showing low risk for reactivation (Demissie et al.; 2004). Health-care workers who were heavily exposed to TB showed intermediate concentrations of neopterin, potentially indicating risk of reactivation of latent M. tuberculosis infection (Ozdemir et al.; 2006).

While a number of biomarkers have been found to be associated with TB protection or TB disease, there are no qualified biomarkers to indicate protection by new vaccines against TB. IFN-γ has been used as a biomarker of protection; however, it is not a reliable biomarker of protection though it is an important component of the immune response. Expression of the cytokine may rather be a marker of the magnitude of the inflammatory response (Mittrucker et al.; 2007). Currently, polyfunctional T cells expressing multiple cytokines (IL-2, TNF-α and IFN-γ) are being increasingly studied; however Kagina et al. recently reported that the specific CD4 T cell response 10 weeks after BCG vaccination in newborns do not correlate with ultimate risk of TB disease. Moreover, risk of disease during the first 2 years

of life was not associated with mono or polyfunctional or CD8 T cells in newborns (Kagina et al.; 2010).

In summary, there is no consensus biomarker(s), which predict protection or progression to disease. Although considerable evidence exists that IFN-γ is involved in immunity, expression of this marker alone has little predictive value. While disease associations with other biomarkers or biomarker combinations have been reported, results have been inconsistent across laboratories, underscoring the need for more research.

1.4 Vaccination strategies against tuberculosis

The current vaccine against TB, Bacillus Calmette-Guérin (BCG) vaccine, which was developed between 1906 and 1919 without any immunological correlation by attenuation of the virulent Mycobacterium bovis, has been given 4 billion times over the last 90 years (Kaufmann; 2010). BCG is given as part of the expanded program of immunization and has an excellent safety record, is inexpensive and has proven protective efficacy against severe childhood forms of the disease, against meningitis and military TB and to a lesser extent against lung TB in infants. It is, however, not effective against pulmonary TB in adults, which is the most prevalent form of the disease. Moreover, in HIV infected infants the risk of disseminated BCG disease is significantly higher (Bricks; 2004). This failure of protection and risk of disseminated BCG in HIV infected children underscore the need for novel improved vaccination concepts towards safe vaccines against newborn and adult TB in HIV negative or positive individuals.

Considering the global TB epidemiology, TB vaccination strategies follow two different approaches: pre-exposure vaccination in order to prevent disease in individuals who have so far not encountered M. tuberculosis versus post-exposure vaccination that aims at inhibiting

disease outbreak in individuals that are already infected (Figure 1-7) (Kaufmann et al.; 2010).

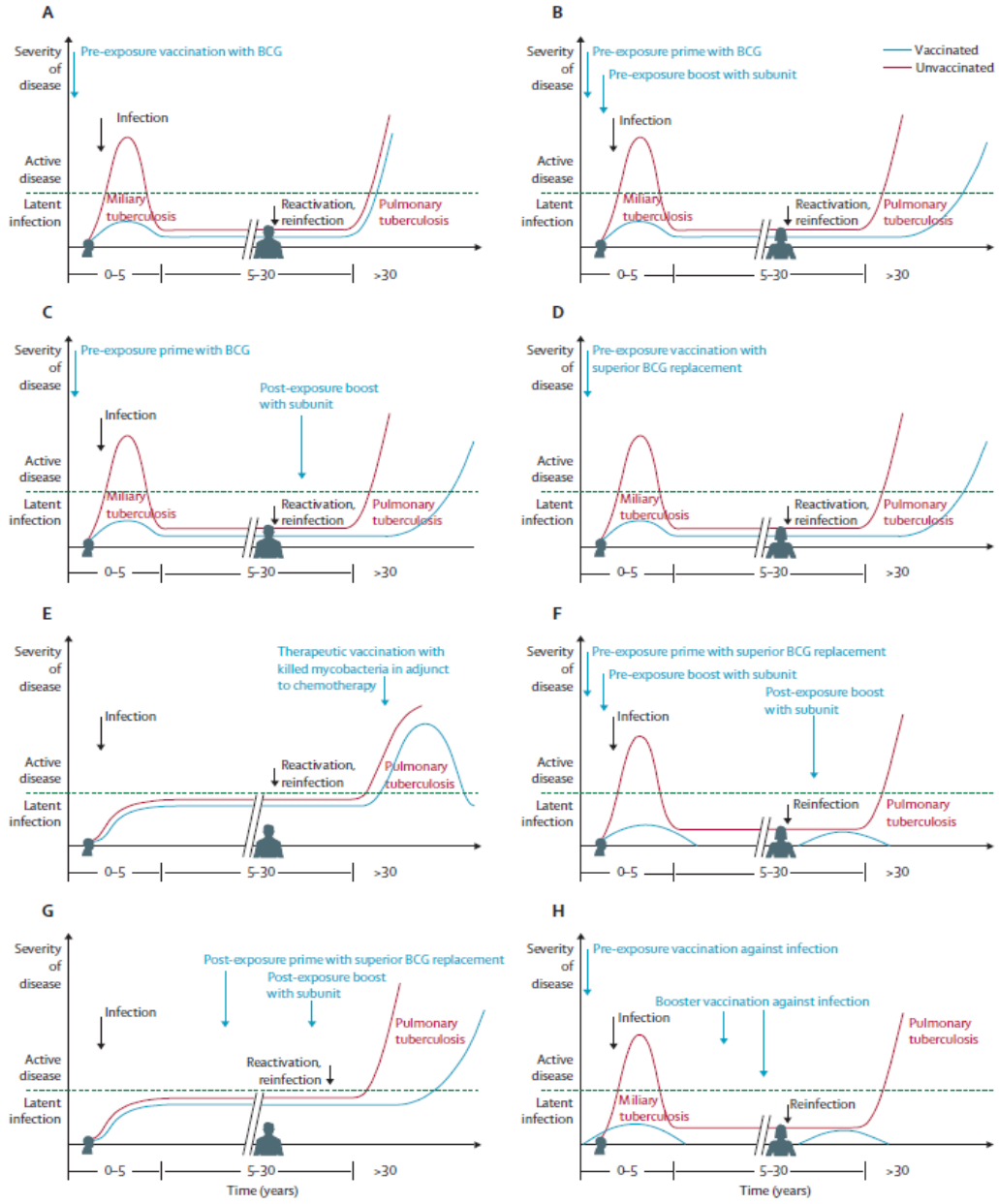


Figure 1-7 Different vaccine strategies (Kaufmann; 2010).
(A) BCG vaccination at birth or pre exposure. (B) BCG prime and pre-exposure boost in children ; (C) Early childhood BCG prime and post-exposure boost in adults; (D) BCG replacement with better vaccine and pre-exposure vaccination at early age; (E) Active tuberculosis cases therapeutic vaccination; (F) BCG replacement vaccine at pre exposure and boost with another subunit vaccine at pre and post exposure; (G) BCG replacement vaccine at pre exposure and boost with another subunit vaccine at post exposure; and (H) pre-exposure and booster vaccination vaccination to prevent stable infection with M. tuberculosis.

The first category follows the approach to improve the current BCG vaccine through recombinant (r) BCG strain constructs with improved vaccine efficacy that are intended to replace BCG. The two major representatives of this group are rBCG30, which is a BCG strain over-expressing the immunodominant M. tuberculosis antigen 85B, and rBCG•UreC:Hly (VPM1002), which is a recombinant strain that is deficient in urease and expresses listeriolysin produced by Listeria monocytogenes (Grode et al.; 2005; Tullius et al.; 2008). This vaccine facilitates the presentation of antigen to CD8 T cells in the context of MHC class I by translocating M. tuberculosis antigen into the cytoplasm via perforating the phagosomal membrane with the help of the acidic phagosomal pH due to deficiency of urase. Another viable vaccine candidate engineered with a similar approach introduces pore-forming capacities into BCG with perfringolysin (pfo) from Clostridium perfringens and expresses antigens Ag85A, Ag85B, and TB10.4 (Sun et al.; 2009).

In contrast, the second category of vaccine candidates is considered more for heterologous prime-boost strategies, with BCG or rBCG as the prime vaccine. The first subgroup includes viral vectors that express immunodominant M. tuberculosis antigens for the initiation of strong Th1-dominated immune response to the expressed heterologous antigen and also induce CD8 T-cell responses. Currently two viral vectors are exploited for TB vaccines: modified vaccinia virus Ankara (MVA) developed by Oxford University and replication-deficient adenovirus (Ad) of serotype 5 or 35 (Ad5 created by McMaster University and Ad35 created by Crucell and Aeras) with the advantage of a strong lung tropism that leads to an increased expression of immunodominant antigens at the site of mycobacterial entry. The MVA and Ad5 virus carriers were both engineered to express Ag85A whereas the Ad35

co-expresses the antigens Ag85A, Ag85B, and TB10.4. Pre-existing antibodies to adenovirus from frequent natural infections could impair Ad-based vaccine efficacy (Kaufmann; 2010).

Fusion proteins of immunodominant antigens with the aim of mounting strong immune responses against immunologically important M. tuberculosis antigens are also used as a heterologous prime-boost strategy. To ensure immunogenicity, recombinant protein vaccines need an adjuvant that promotes Th1 immune responses. Three different types of adjuvants are currently used for protein vaccines. Hybrid1 (H1) which is a fusion of the antigens 85B and ESAT-6 or HyVac4/AERAS-404, which is a fusion protein of Ag85B and TB10.4 (Dietrich et al.; 2005; van Dissel et al.; 2010) has been used in combination with an adjuvant IC31 developed by Intercell. In the M72 vaccine, the antigens Rv1196 and Rv0125, are supplemented with adjuvants AS01 or AS02 (Von Eschen et al.; 2009). The inactivated mycobacteria M. vaccae, an environmental mycobacterium, and the semi-purified M. tuberculosis fragments RUTI are considered as therapeutic vaccinations that could potentially synergize with chemotherapy (Vilaplana et al.; 2010; von Reyn et al.; 2010). M. vaccae is a whole-cell vaccine thought to mount a protective immune response by providing cross-reactive antigens. RUTI comprises detoxified and fragmented M. tuberculosis components carried in liposomes.

On top of the aforementioned vaccines which are already in clinical trials, there are also other future strategies which aim to induce long-lasting memory T cell responses comprising mostly CD4 Th1 cells that resist exhaustion, suppression, and deviation. This strategy targets CD4 cells to remain in a stage of alertness, whereby immune mechanisms can be promptly mobilized after encounter with M. tuberculosis or vaccines that achieve sterile eradication

of the pathogen in latently infected individuals or protect naive individuals by rapid elimination of M. tuberculosis after infection (Kaufmann; 2010).

1.5 Significance of the study

Tuberculosis continues to be a major global health problem, causing an estimated 8.8 million new cases and 1.45 million deaths annually despite the availability of a vaccine and inexpensive, effective, and reasonably well-tolerated therapy. Although WHO declared TB as a global emergency in 1993 and a number of efforts have been in place, the situation did not change significantly and TB remains the second most important killer disease next to HIV/AIDS. We are still using very old diagnostic techniques particularly in developing countries where the disease burden is high, old drugs with 6 month of therapy, which makes adherence challenging and a vaccine, which is not at all protective in some parts of the world.

The United Nations and the Stop TB Partnership aimed to decrease TB prevalence by half in 2015 as compared to 1990 and to lower the incidence of new cases to less than 1 per million by 2050 (Dye and Williams; 2008). Albeit the TB prevalence is decreasing globally, it is unlikely any of these targets will be reached with the available diagnostic, therapeutic and preventive methods. Therefore, it is essential to develop better diagnostic tools, new drugs for active cases and latently infected individuals and vaccines for latently infected and uninfected individuals as well as HIV positive newborns.

A recent mathematical model prediction showed that In 2050 TB incidence shall be reduced up to 71% if new vaccines, drugs and diagnostic tools are developed and introduced with a potential of each to reduce TB incidence by 39-52%, 10-27% and 13-42% respectively (Abu-Raddad LJ et al.; 2009). It has been also postulated that the incidence of TB could be

reduced up to 94% in 2050 and the 2050 target could be reached by applying mass vaccination and introducing new diagnostic tools (Kaufmann et al.; 2010) .

Therefore, in order to achieve the very ambitious targets of the Millennium Development Goals and curb the current TB problem, explorative studies on transcriptomic, proteomic and immunologic profiling, which could provide clues for developing new diagnostic tools, therapeutics and vaccines are more critical than ever before and will have a significant impact on the global tuberculosis problem. These exploratory studies would help in identifying and developing simple, affordable and rapid methods for detecting active cases, discriminating between and diagnosing active tuberculosis disease and latent tuberculosis infection and identifying persons most likely to progress to active disease and relapse after treatment. This is particularly true for developing countries where the incidence of active and latent tuberculosis is high and where smear microscopy is the only technique widely used for active case detection. A reliable test for latent infection would be valuable to guide interventions in those most likely to progress to active TB, including HIV infected people and young children.

Moreover, understanding the genetic diversity of M. tuberculosis strains is also an important component in designing new tools as some studies showed that strain differences affect effectiveness of drugs and vaccines and cytokine responses induced during infection of human macrophages.

1.6. Hypothesis

We hypothesized that in TB cases and their contacts there will be:

a) Discriminatory transcriptional signatures of immune response genes. Combinations of multiple genes could potentially distinguish TB cases and their contacts and the different clinical outcomes of M. tuberculosis infection.

b) Different plasma cytokine levels in TB cases and their contacts. Combination of the different cytokines could be potential biomarkers for discriminating TB cases and their contacts. HIV co-infection could affect the plasma level of these cytokines. These plasma levels of cytokines in TB cases will also be affected by anti tuberculosis treatment and will be similar to patterns in contacts after completion of treatment.

c) Both 'modern' and 'ancient' genotypes of M. tuberculosis isolates are found in Addis Ababa as historical evidence showed that TB may have originated in the region and their proportion could be similar to that observed in other countries in the region and

d) The genotype of M. tuberculosis will affect the plasma cytokines patterns in TB patients infected with different strains.

1.7 Aims

The main objectives of this study are:

- To study selected transcriptional signatures of different genes in whole blood in tuberculosis patients and healthy household contacts with Multiplex Ligation Dependent Probe Amplification (MLPA) and to identify genes or combination of

genes which have discriminatory potential between TB cases and their contacts and between latently infected and uninfected contacts;

- To determine the plasma levels of cytokines in TB cases and their contacts and identify cytokines or combination of cytokines which have a discriminatory potential and could be used as biomarkers in differentiating between TB cases and their contacts and between latently infected and uninfected individuals;

- To characterize the genotypes of M. tuberculosis isolates which are circulating in Addis Ababa, Ethiopia;

- To determine the effect of infecting M. tuberculosis strains on the plasma level of cytokines in tuberculosis patients.

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CHAPTER 2

2. Materials and Methods

2.1 Study Area

The study was conducted in Addis Ababa, Ethiopia. Ethiopia is a country located in the horn of Africa. It is the second-most populous nation in Africa, with over 82 million inhabitants, and the tenth largest by area, occupying 1,100,000 km^2. Ethiopia is bordered by Eritrea to the north, Djibouti and Somalia to the east, Sudan and South Sudan to the west, and Kenya to the south. It is also the most populous landlocked nation in the world. Addis Ababa is the capital city of Ethiopia and often referred to as "the political capital of Africa", due to its historical, diplomatic and political significance for the continent. The city lies at an altitude of 7,546 feet (2,300 metres) and is located at 9°1•48•N 38°44•24•E. Addis Ababa has a subtropical highland climate. The city possesses a complex mix of highland climate zones, with temperature differences of up to 10 °C. According to the 2007 census conducted by the Central Statistical Agency of Ethiopia, the city has a total population of 2,739,551 of whom 1,305,387 are men and 1,434,164 are women (Ethiopia; 2007). The study was conducted in four selected health centres that fall under the administration of the Addis Ababa Health Bureau: Arada, T/Haimanot, Kirkos and W-23 health centres.

2.2 Study Design and Study Period

The study was cross-sectional, conducted between March 2009 and December 2010 among newly diagnosed TB patients who presented to the health clinics and their contacts living in Addis Ababa, the capital city of Ethiopia, where the incidence rate of TB is high and BCG vaccination is routinely administered at birth. A longitudinal study was conducted on a subgroup of the TB cases.

2.3 Study Population

The study population comprised two groups of study participants from the catchment areas of selected health centres who were recruited based on the presence or absence of active tuberculosis infection. The study population consists of male and female new index pulmonary TB patients and their household contacts aged between 18-60 years.

Index Cases (ICs)

Any tuberculosis suspect case attending the health centres for symptoms (including persistent cough, fever, haemoptysis, night sweating) suggestive of pulmonary tuberculosis and diagnosed by sputum smear examination (AFB positive in at least 2 out of 3 spot-morning-spot sputum samples).

Household contacts

These were apparently clinically healthy contacts of index cases living under the same roof of the index case for at least two weeks in the period of 3 months prior to tuberculosis diagnosis of the index case.

2.4 Laboratory Methods

2.4.1 Blood Collection

Twenty ml of venous blood was drawn from all eligible participants, where about 3 ml of venous blood was drawn into QuantiFERON tubes (Cellestis, Australia), 2.5 ml into PAXgene blood RNA tubes (PreAnalytiX, QIAGEN, Germany) and the rest of the blood into sodium heparin tubes for isolation of plasma and peripheral blood mononuclear cells (PBMCs). After collection, all blood specimens were transported to the AHRI laboratory at ambient

temperature and kept at room temperature for no more than 2 hours until processing, except for QuantiFERON tubes, which were incubated at 37^{O}C immediately upon arrival. Plasma was collected from blood collected in heparinized tubes by centrifuging for 5 min at 1000 RPM, aliquoted and stored at -80°C until cytokine determination.

2.4.2 Quantiferon Gold In Tube (QFT- GIT) TB test

IFN-γ responses to M. tuberculosis specific antigens were measured by the QFT assay, as per the manufacturer's instructions, (http://www.cellestis.com). Three ml venous blood was directly collected into three 1-ml QuantiFERON-TB Gold In Tube (QFT-GIT) tubes (Cellestis, Victoria, Australia); one tube containing only heparin, a negative control (Nil), another tube containing phytohaemagglutinin (PHA), a positive control (Mitogen) and the third tube containing overlapping peptides representing the entire sequences of ESAT-6, CFP-10 and TB7.7 (TB Antigen). The tubes were shaken vigorously and then incubated at 37°C for approximately 20 hours. After 20 hours of incubation, the tubes were centrifuged and plasma was harvested and frozen at -20 C until the ELISA was performed. The level of IFN-γ was measured using the QFT ELISA kit (Cellestis, Victoria, Australia). The ELISA readout and data interpretation was carried out using the QFT software (Version 2.50, Cellestis, Australia. As recommended by the manufacturer, a positive test for TB infection was considered if the IFN-γ difference was $\geq$0.35 IU/ ml (TB antigens -Negative control). The result of the test was considered indeterminate if an antigen-stimulated sample was $\leq$ 0.35 IU/ml (TB antigens-Negative control) plus if the value of the positive control was less than 0.5 IU/ml (Positive control-Negative control).

2.4.3 Multiplex Cytokine analysis

We used a 17plex kit (Epidermal Growth Factor (EGF), FRACTALKINE, Granulocyte Macrophage Colony Stimulating Factor (GM CSF), IFN-γ, IL-1, IL 10, IL-12, IL-17, IL-4, IL-7, IL-9, IFN-γ inducible protein (IP-10 /CXCL-10), Macrophage Chemoattractant Protein 1 (MCP-1/CXCL), MCP-3, Monocyte Inflammatory Protein 1 beta (MIP-1β), TNF and VEGF) from Millipore, Germany and multicytokine analysis was done using Luminex (Millipore, Germany) technology. Cytokines that have been associated with active TB disease or protection against disease in literature were included in the study. The principle of the technique is based on colour coded microspheres where combining different ratios of two dyes can give up to 100 different combinations, which enables the measurement of up to 100 analytes. The technology combines flow cytometric and ELISA principles. Capture antibody is conjugated with beads or microspheres whereas the secondary antibody is conjugated with a flurochrome, which quantifies the antigen-antibody reaction by measuring the relative fluorescence intensity. The assays were performed according to the supplier instruction. Briefly, following pre wetting of plates, 50 µl precombined beads of all the 17 individual anti-cytokines or anti-chemokines antibodies were added and washed twice. Plasma samples (25µl) were diluted 1:1 with the kit serum matrix and added to the plate. The plate was shaken for 30 sec at 1000 RPM and then incubated for 1 hour on a plate shaker at 300 RPM at room temperature. Plates were washed twice and 25ul of detection antibody was added per well and incubated for one hour on a plate shaker. Fifty micro litre of a strepatavidin-PE conjugate was added per well and incubated for 30 min at room temperature. Finally, plates were washed three times and 150 microliter of sheath fluid was

added to each well and then the plate was read on a Luminex machine and data was analysed by Luminex 100 IS software version 2.3.182.

2.4.4 RNA Extraction

RNA extraction was performed following the manufacturer's instructions (PAXgene Blood RNA Kit, PreAnalytiX, QIAGEN)(http://www.mlpa.com). Briefly, blood containing tubes were centrifuged at 3000 rpm for 10 min, supernatant discarded, pellets lysed and washed, followed by treatment with proteinase K and ethanol precipitation. To remove contaminating DNA, the analyte was further digested with RNase-free DNase (QIAGEN, Germany), washed and finally the RNA was eluted with RNase-free water, concentrations quantified using GeneQuant spectrophotometer (Amersham Biosciences, UK) and stored at -80^{o}C until use.

2.4.5 Multiplex Ligation-dependent Probe Amplification

Multiplex Ligation-dependent Probe Amplification (MLPA) was done according to the manufacturer instruction (http://www.preanalytix.com). The principle of MLPA follows cDNA synthesis from mRNA and denaturation; hybridization of probes with the target genes; ligation of the two separate probes; PCR amplification; separation of the PCR product and data analysis (Figure 2-1). First, the cDNA is synthesised from RNA by using RT primer mix and cDNA denatured and incubated overnight with the mixture of customized probes to allow the probes to hybridize with the target genes. Once the probes are hybridized with the target genes, the two separate probes are fused together using a ligase enzyme. The target genes, which were hybridized with the probes, are amplified. Finally the PCR product is separated by electrophoresis and the data analysed by measuring the fluorescence intensity.

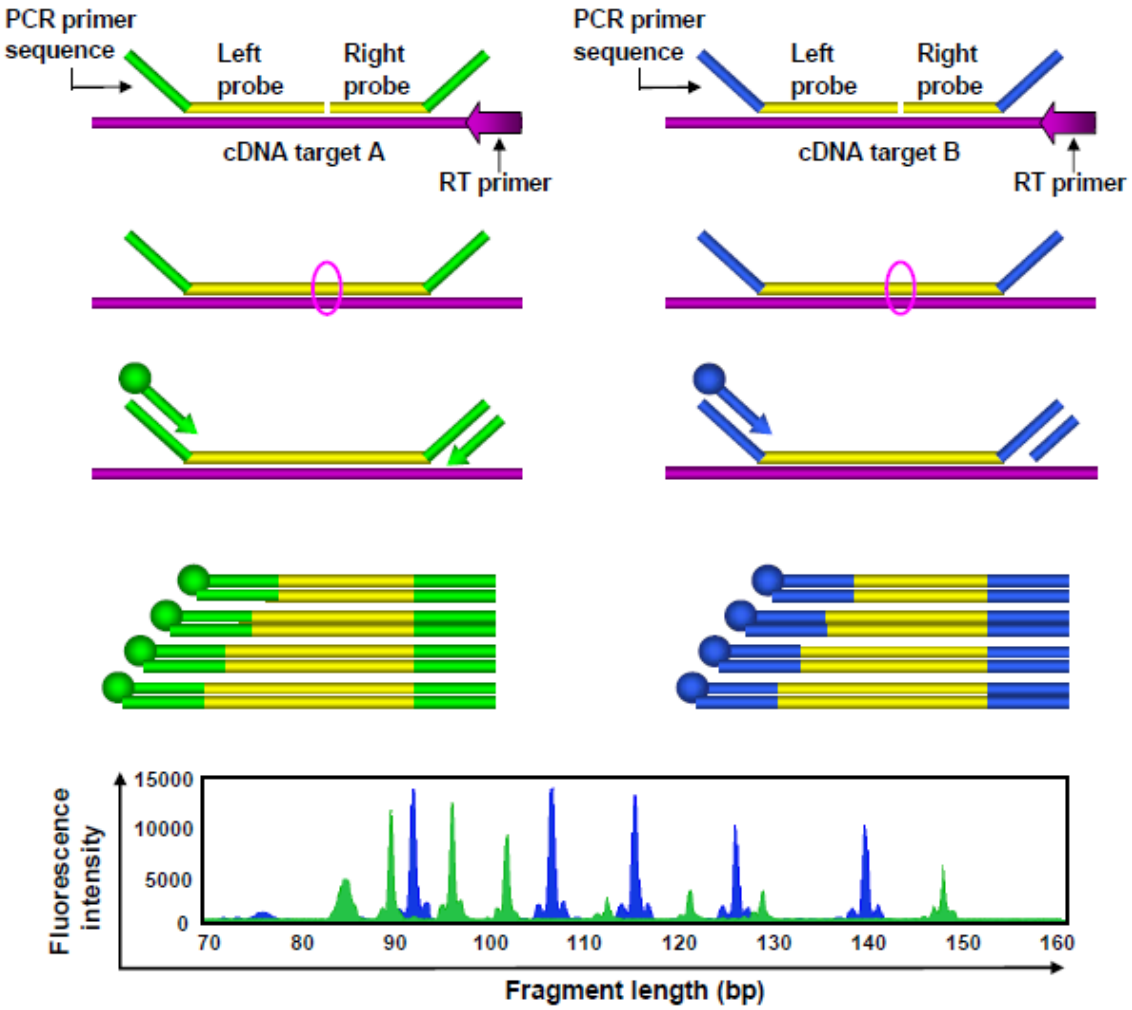

Figure 2-1 Multiplex Ligation dependent Probe Amplification (Joosten 2012).
RNA samples are reverse transcribed to cDNA with Reverse transcriptase (RT) enzyme. Denatured genomic cDNA is hybridized with a mixture of probes. Each MLPA probe consists of two oligonucleotides. The two parts of each probe hybridize to adjacent target sequence on the cDNA. The two parts of hybridized probes are ligated by a thermostable ligase. All probe ligations are amplified by PCR using two different primer pairs, each labelled with a different fluorophore. The amplification product of each probe has a unique length. Amplification products are separated by electrophoresis. Relative amounts of probe amplification products reflect the relative copy number of target sequences.

A probe set has been designed for the GC 6-74 project by the Leiden University Medical Centre (LUMC), Leiden, The Netherlands and this set of probes contains forty five genes targeting immune cell subset markers, T reg markers, effector T cell markers and apoptosis related genes and four housekeeping genes. Genes that have been associated with active TB

disease or protection against disease in literature were included in the study. The list of genes for which a set of probe has been designed and studied for the GC 6 project is shown in table 2-1.

Table 2-1 List of target genes for Multiplex Ligation-dependent Probe Amplification (MLPA)

Bcl2	CD8•	IL-4	RAB33
BLR1(CXCR5)	CD14	IL-4•2	ASEC14-like 1
BPI	CD19	IL-7R (CD127)	SPP1
CASP8	CD163	IL-10	TGF•1
CCL4	CTLA4 (CD152)	IL-22RA1	TGF•-RII
CCL13	CXCL10 (IP-10)	LAG3	TNF
CCL19	FASLG	LTF	TNFRSF1A
CCL22	Fc•R1A (CD64)	MARCO	TNFRSF1B
CCR7	FOXP3	MMP9	TIMP2
CD3•	FPR1	NCAM1 (CD56)	TNFRSF18 (GITR)
CD4	IFN •	RAB13	Reference genes
	IL-2R• (CD25)	RAB24	ABR, •2M, GAPDH, GUSB

Multiplex ligation-dependent probe amplification was performed following the instructions provided by MRC Holland, Amsterdam, The Netherlands (http://www.mlpa.com). Briefly, 2.5 µl RNA sample mixed with 1 µl MMLV buffer plus 1 µl RT primer mix was heated at 80 for 1 minute and then incubated at 45°C for 5 minutes. While the mix was in the

thermocycler, the temperature was lowered to 37°C and 1.5 µl of MMLV reverse transcriptase was added and incubated for 15 minute. The reverse transcriptase was inactivated by heating to 98°C for 2 minutes and then after cooling to room temperature mixed with 3 µl of probe mix (containing 1.5 µl of each probe) and 1.5 µl of SALSA hybridization buffer, denatured at 95°C for 1 min and hybridized at 60°C for 16 h. Hybridized probes were ligated at 54°C for 15 min by addition of 32 µl ligation mixture (3 µl ligase 65 buffer A, 3 µl ligase 65 buffer B, 1 µl of 65 ligase and 25 µl water). Following heat inactivation at 98°C for 5 min, 5 µl of ligation reaction was mixed with 20 µl of PCR master mix (2 µl SALSA PCR buffer, 1 µl SALSA enzyme dilution buffer, 1 µl SALSA PCR primers MLPA, 2 µl LUMC PCR primers MAPH, 0.25 µl SALSA polymerase and 13.75 µl water) and was subjected to PCR amplification for 33 cycles (30 s at 95°C, 30 s at 58°C, and 1 min at 72°C) and ended with 20 minutes incubation at 72°C. A negative control (without RNA), a positive control (using synthetic template oligonucleotides as hybridization templates) and a commercial Human Universal Reference RNA were included on each run.

After completion of the MLPA reaction, amplified products were analysed with an ABI-310 capillary sequencer in GeneScan mode (Applied Biosystems). The data from the sequencer were analysed using the GeneMapper software. Further analysis was done using Microsoft Excel spread sheet software. Finally data was normalised by selecting one of the housekeeping genes, which most stably expressed across the evaluated samples (ABR, GUSB, GAPDH or B2M). The coefficient of variation was calculated to determine which reference gene was most stably expressed across the evaluated samples. A peak area of 200 for signals was assigned as threshold value for noise cut off in GeneMapper. The relative

peak size of the product from the probe recognition sequence was compared with the relative peak size of the product from a control.

2.4.6 Mycobacteria Culture and DNA extraction

Two sputum samples were collected from each participant and pooled together for culture. An equal volume of 4% NaOH was added and shaken using an automatic shaker for 15min. The supernatant was decanted after centrifuging at 3000rpm for 15 min. One to two drops of phenol red indicator was added and then neutralized by using 2N HCL by adding drop by drop and shaken continuously until the colour changed from red to yellow. Once the sputum sample was decontaminated and neutralized, 100 µl was inoculated on Loewenstein Jensen media. The inoculated cultures were incubated in a slant position for one week at 37 ^{o}C and then turned upright in the second week and followed up for growth over a period of 8 weeks. Cultures with no growth after the eighth week were considered negative. Mycobacterial genomic DNA was isolated by heating the isolates at 80°C for 60 min and was stored at -20°C until it was subjected to spoligotyping.

2.4.7 Deletion Typing

Isolates were confirmed as M. tuberculosis by deletion typing of the RD9 region according to a previously described PCR protocol (Brosch et al.; 2002b). The status of the RD9 region (deleted or intact) was assessed by multiplex PCR with a set of three primers (primer set RD9): two primers targeting the flanking regions of RD9 (RD9_Fw, 5'- AAC ACG GTC ACG TTG TCG TG -3', RD9_Rev, 5'- CAA ACC AGC AGC TGT CGT TG -3'and one primer hybridizing with the internal region of RD9 (RD9_IntRev, 5'- TTG CTT CCC CGG TTC GTC TG -3'. A PCR product

of 396 bp interpreted as that RD9 is present (i.e. M. tuberculosis) or 707 bp (RD9 deleted i.e. M. africanum and M. bovis) was identified by agarose gel electrophoresis (Figure 2-2).

The PCR mixture used for RD contained 2 •l of heat-killed mycobacterial DNA, a final concentration of 10 µl HotStartTaq master mix (Qiagen), 0.3 µl primers RD9_FW, RD9_Rev, and RD9_IntRev (100 µM), and 7.1 µl sterile distilled water to a final volume of 20 •l. Thermal cycling was performed with T-3000 Thermocycler Biometra Amplifier (Biometra, Göttingen, Germany) with an initial denaturation step of 15 min at 96°C, 35 cycles of 30 s at 96°C, 30 s at 55°C, and 1 min at 72°C, followed by a final elongation step of 10 min at 72°C. PCR products were separated on a 1% agarose gel.

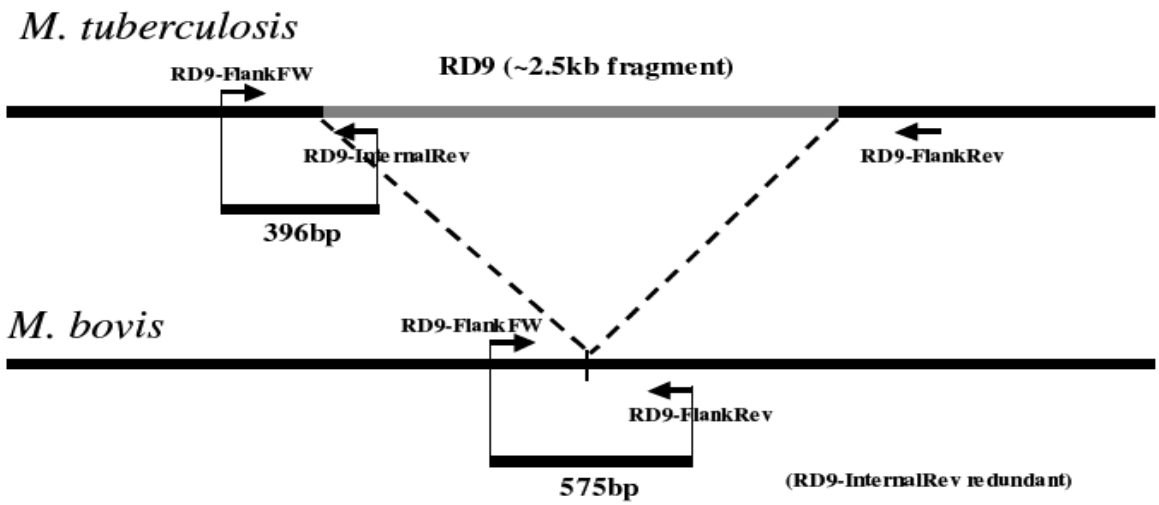

Figure 2-2 Deletion typing of the RD9 region in the genome of M. tuberculosis and M. bovis, respectively.
In case of M. tuberculosis, RD9-Ff and RD9Int will amplify a product size of 1.5Kbp due to the presence pf RD9; whereas in case of M. bovis since RD9 is deleted, RD9Ff and RD9Fr will amplify a product size of 472Bpb.

2.4.8 Spoligotyping

Spoligotyping is a PCR based molecular technique based on DNA polymorphism at the genomic Direct Repeat (DR) locus of Mycobacterium tuberculosis complex. The direct repeat locus consists of a repeated 36 base pair sequences and are separated by non-repetitive 35-41 base pair length DNA spacers (figure 2-3). Although there are about 94 different spacers in the M. tuberculosis complex, only the originally described 43 spacers are used routinely for the classification of strains. One DR and its neighboring spacer are termed a "Direct Variant Repeat" (DVR). It was observed that the order of the spacers is about the same between clinical isolates, although the DR region has been shown to be polymorphic in different clinical isolates of M. tuberculosis. There are one or more IS6110 elements in most strains of M. tuberculosis in the DR region.

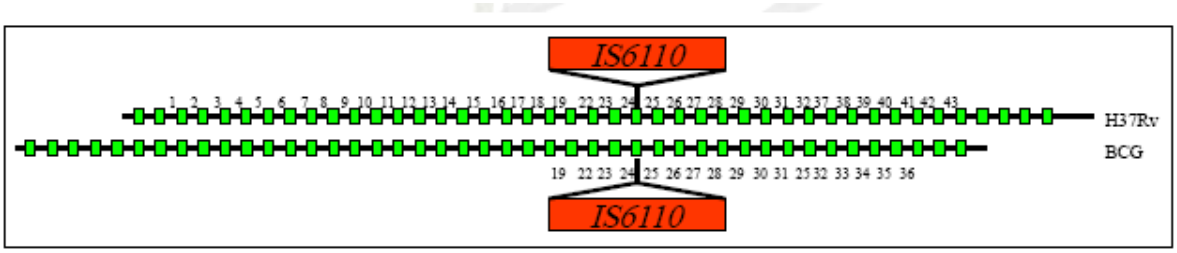

Figure 2-3 Structure of the DR locus in the genome of M. tuberculosis H37Rv and M. bovis BCG. The green rectangles depict the 36 bp Direct Repeat (DR).

Spoligotyping was carried out using the commercially available kit from Ocimum Biosolutions, India, according to the manufacturer's instructions (Kamerbeek et al.; 1997b) . Briefly, the direct-repeat (DR) region was amplified with primers DRa (50-GGT TTT GGG TCT GAC GAC-30 biotinylated at the 5' end) and DRb (50-CCG AGA GGG GAC GGA AAC-30). PCR amplification was done for 53 cycles with denaturation, and annealing extension for 1min at 95 ^{0}c and 55 ^{0}c respectively and extension for 30 sec at 72^{0}c in each cycle (figure 2-4). The

amplified DNA was hybridized to inter-DR spacer oligonucleotides covalently bound to a membrane. Sterile water and DNA from M. tuberculosis and M. bovis were used as negative and positive controls respectively. The amplified DNA was subsequently hybridized to a set of 43 oligonucleotide probes by reverse line blotting. The hybridized PCR products were incubated with streptavidin-peroxidase conjugate, and signal detection was obtained with an enhanced chemiluminescence detection system (Amersham, Little Chalfont, England), followed by exposure to X-ray film (Hyperfilm ECL; Amersham) according to the manufacturer's instructions. The X-ray film was developed and washed using standard photochemical procedures.

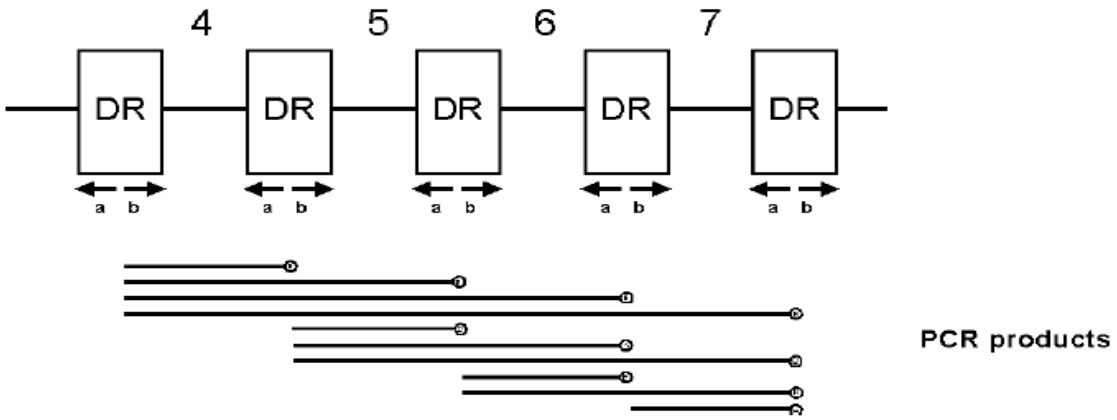

Figure 2-4 Principle of DNA amplification of the DR region of M. tuberculosis complex bacteria.
The two primers, a and b will lead to the amplification of any spacer or a stretch of neighboring spacers and DR's.

2.4.9 Database comparison

A Microsoft Excel sheet was prepared and the spoligotyping results were entered manually in binary format and then the binary format was converted into octal form. The black and white squares in the spoligopattern represent the presence and absence of individual

spacers respectively. The spoligopatterns, which were prepared in binary and octal formats were entered and determined by comparing the spoligotyping results with already existing designations in the international spoligotyping database, SpolDB4.0 (Brudey et al.; 2006) (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo). In this database, shared types (ST) designates spoligotypes shared by two or more strains having identical spoligopattern whereas single patterns which do not have a match in the database are designated as Orphans. There are 62 lineages and sub lineages defined in the SPOIDB4 database specific for the members of M. tuberculosis complex species. Currently, the SpolDB4 database has a collection of about 39, 609 spoligopatterns from 121 countries all over the world. Families and subfamilies were assigned for patterns that were not found in SpolDB4.0 data base using SpotClust program (http://cgi2.cs.rpi.edu/ bennek/SPOTCLUST.html) (Vitol et al.; 2006).

2.5 Ethical considerations

The study protocol was approved by the AHRI/ALERT (P015/10) and National Ethical Review Committees (3.17/17/03). Written informed consent was obtained from all study participants. The informed consent described the purpose of the study, the procedures to be followed, and the risks and benefits of participation. All records were kept in a secured place and copy of the consent was given to all participants.

2.6 Statistical Analysis

The data were analyzed using Graph pad prism software, version 4.0 for Windows, GraphPad Software, San Diego California USA, and STATISTICA software, version 10. Nonparametric Mann–Whitney U tests were performed to find the significance of the

observed differences between the different groups. STATISTICA multivariate analysis for General Discriminant Analysis (GDA) and Receiver Operator Characteristic (ROC) curve were used to evaluate the predictive abilities of combinations of biomarkers and generate cut off values for differentiating between M. tuberculosis infection states. The GDA is a multivariate analysis of variance hypothesis of the test that two or more groups (conditions, levels) differ significantly on a combination of discriminating variables whereas ROC, or ROC curve, is a graphical plot which illustrates the performance of a binary classifier by plotting the fraction of true positives out of the positives (Sensitivity) vs the fraction of false positives out of the negatives (one minus the specificity). A P value less than 0.05 was considered as statistically significant.

2.5 References

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CHAPTER 3

3. Expression of immune response genes from whole blood discriminate the different clinical tuberculosis groups in Ethiopian cohorts

Abstract

Background: Genetic factors are involved in susceptibility to tuberculosis. Apart from gene polymorphisms and mutations, changes in levels of gene expression, induced by non-genetic factors, may also lead to differences amongst individuals in protection from or progression to tuberculosis.

Methodology: We analysed the expression level of 45 genes from whole blood in a total of 47 individuals (23 healthy household contacts and 24 microbiologically confirmed new smear positive pulmonary TB patients) in Addis Ababa using a dual colour multiplex ligation-dependent probe amplification (MLPA) technique to assess gene expression profiles that may be used to distinguish TB cases and their contacts and also latently infected and uninfected household contacts

Results: The gene expression level of BLR-1, Bcl-2, IL-4d2, IL-7R, FcgR1A, MARCO, MMP-9, CCL-19, and LTF have sufficient discriminatory power between sputum smear positive TB cases and household contacts with AUCs of 0.84, 0.81, 0.79, 0.79, 0.78, 0.76, 0.75, 0.75 and 0.68 respectively. The combination of Bcl-2, BLR-1, FcgR1A, IL-4d2 and MARCO identified 95.83% of active TB cases and 95.65% of household contacts without TB. The expression of CCL-19, TGFb1, and Foxp3 showed significant differences between latently infected and uninfected contacts with AUCs of 0.85, 0.82, and 0.75 respectively whereas the combination of BPI, CCL-19, FoxP3, FPR1 and TGFb1 identified 90.9% of QFT negative and 91.6% of QFT positive household contacts.

Conclusion: Expression of single and especially combinations of genes can accurately differentiate between active tuberculosis cases and non-tuberculosis cases as well as between latently infected and uninfected contacts.

3.1 Introduction

An effective immune response controls infection with M. tuberculosis in a majority of infected individuals and it is only 10% of the infected who will develop clinical disease and symptoms within the first two years after infection (primary TB) and another 5% develop the disease later in life (reactivation TB). This raises the question ‘‘what is different about those who control tuberculosis infection and who fail to control tuberculosis infection?’’ Many studies have confirmed that genetic factors are involved in the disease and could be a key difference in the different outcomes of M. tuberculosis infection (Möller and Hoal; 2010). A recent study showed a significant difference in the type and magnitude of immune responses against BCG between UK and Malawi children where Th1 related cytokines were present at higher levels in the UK infants whereas the innate proinflammatory cytokines, regulatory cytokines, interleukin 17, Th2 cytokines, chemokines, and growth factors were higher in the Malawi infants, which could be due to genetic but also environmental factors (Lalor et al.; 2011).

Apart from gene polymorphism and mutation which leads to differences among individuals (Sakai et al.; 2001; Awomoyi et al.; 2002; Kim et al.; 2003; López-Maderuelo et al.; 2003; Cooke et al.; 2006; Ates et al.; 2008), there are also changes in levels of gene expression when cells migrate to and from sites of M. tuberculosis infection and interact with mycobacteria, which could also lead to differences in susceptibility (Jacobsen et al.; 2007; Mistry et al.; 2007; Lu C et al.; 2011; Maertzdorf et al.; 2011). Therefore, upregulated or downregulated genes may aid as biomarkers to distinguish the different clinical tuberculosis infection groups. Protective immunity against tuberculosis is associated with Th1 dominated cytokines; particularly IFN-γ, however, different studies have indicated that the level of IFN-γ

alone does not constitute a correlate of protection although its presence is important (Dockrell; 2007; Lalvani and Millington; 2008). Therefore, additional biomarkers, which clearly indicate protection or susceptibility against TB are much needed. Recent studies have indicated that Fc gamma receptor 1B (FCgR1B) (Maertzdorf et al.; 2011), combined expression patterns of FCgR1A (CD64), RAB33A and LTF (lactoferrin) (Jacobsen et al.; 2007) and CD3e, CD8a, IL7R, BLR1, CD19, FCGR1A, CXCL10, CD4, TNF, BCL2, MMP-9, Foxp3, CASP8, CCL-4, TNRFSF1A, CASP8, Bcl2 and TNF (Joosten et al.; 2012) show discriminating power between TB and LTBI. Expression of RIN3, LY6G6D, TEX264, and C14orf2 genes discriminate active, cured, recurrent or latent TB (Mistry et al.; 2007). Therefore, we analysed the expression level of 45 genes targeting immune cell subset markers, T regulatory markers, effector T cell markers and apoptosis related genes and four housekeeping genes using a dual colour multiplex ligation-dependent probe amplification technique to assess gene expression profiles to distinguish the different clinical tuberculosis groups. These markers were chosen by the laboratory of Prof Tom Ottenhoff for a MLPA gene set, which was based on current literature and work in their laboratory.

3.2 Materials and Methods

A total of 47 subjects (23 healthy household contacts and 24 microbiologically confirmed new smear positive pulmonary TB patients) attending Arada, T/Haimanot, Kirkos and W-23 health centres in Addis Ababa were recruited. The diagnosis of TB in the health centres was based on the national guidelines of at least two positive smears for acid-fast bacilli (AFB) in three specimens collected from each patient as spot-morning-spot samples. All sputum samples from TB cases were cultured following the protocol described in the methodology section (2.4.6).

Quantiferon-Gold In Tube (QFT-GIT)

Latent TB infection was detected by performance of QFT-GIT as per the protocol described in the methodology section of the thesis (2.4.2).

Blood collection and RNA Extraction

Blood was collected with PAXgene tube and RNA extraction was performed following the manufacturer's instructions (PAXgene Blood RNA Kit, PreAnalytiX, QIAGEN) as described in the methodology section of the thesis (2.4.4).

Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA was performed following the protocol described in the methodology section of the thesis (2.4.5).

Target Genes

A probe set has been designed for the GC 6-74 TB biomarker project by the Leiden University Medical Center (LUMC), Leiden, The Netherlands and this set of probes contains primers for forty five genes targeting immune cell subset markers, T regulatory cell markers, effector T cell markers, apoptosis related genes and four housekeeping genes. A list of these genes is shown in the methodology section (table 2.1).

Statistical analysis

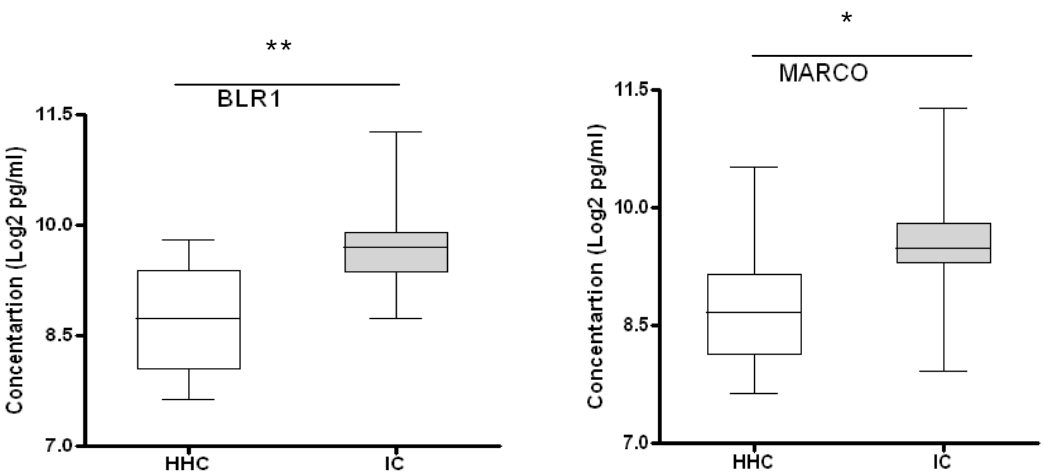
The data were analyzed using Graph pad prism software, version 4.0 and STATISTICA software, version 10. The nonparametric Mann–Whitney U test was performed to assess the significance of the observed differences. General discriminant analysis (GDA) and receiver operator characteristic (ROC) curve analysis were used to evaluate the predictive abilities of combinations of biomarkers and generate cut-off values for differentiating between M. tuberculosis infection states. A P value less than 0.05 was considered as statistically significant.

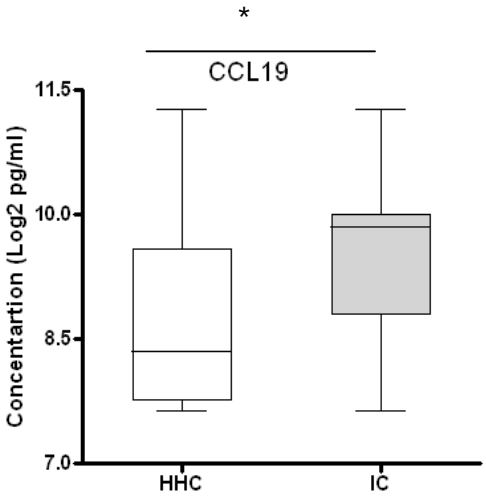
3.3 Result

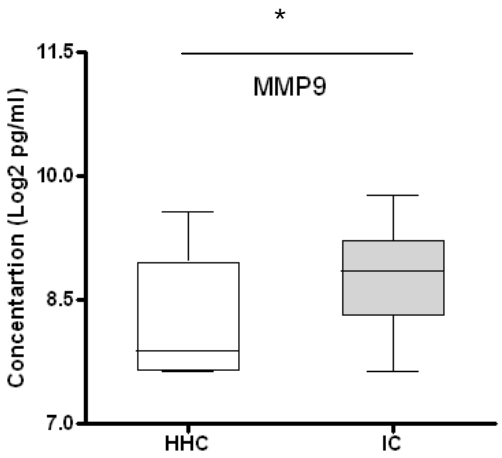
We enrolled 24 subjects with active tuberculosis (12 HIV positive and 12 HIV negative) and 23 HIV negative household contacts comprising 12 QFT-GIT positive and 11 QFT-GIT negative household contacts. The mean age of TB patients was 31.6 ± 1.4 and 46.5% of the participants were females. The mean age for household contacts was 28.3±2.3 and 47.6% of household contacts were females. All HIV positive TB patients were ART naïve with a mean CD4 count of 326 ±31.2 cells/•L.

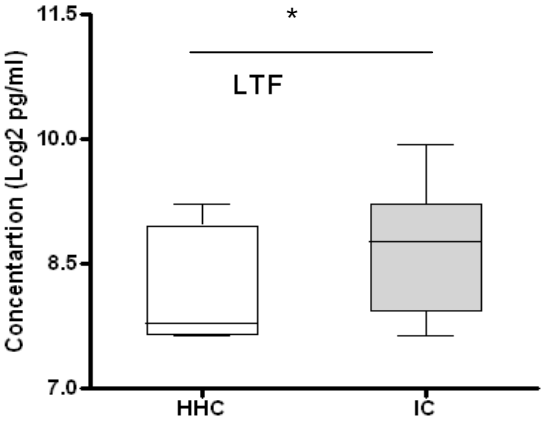
3.3.1 Gene expression of TB patients and household contacts

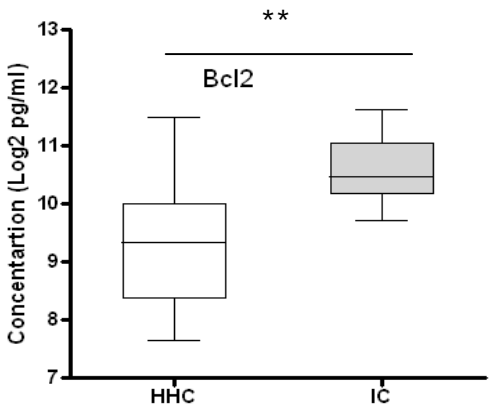
RNA samples from 24 TB patients and 23 healthy household contacts were analysed with MLPA and a significant gene expression difference was observed between these two groups. The gene expression levels of BLR-1, MARCO, CCL-19, MMP-9, LTF, Bcl-2, and FCgR1A were statistically higher in TB patients than contacts ($p<0.05$), whereas the expression level of IL-4d2 and IL-7R were statistically higher in healthy contacts than TB cases ($p<0.05$) (Figure 3-1).

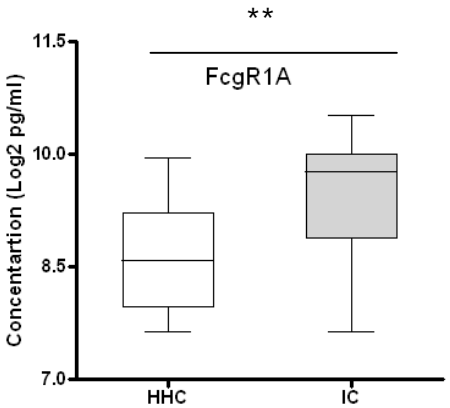












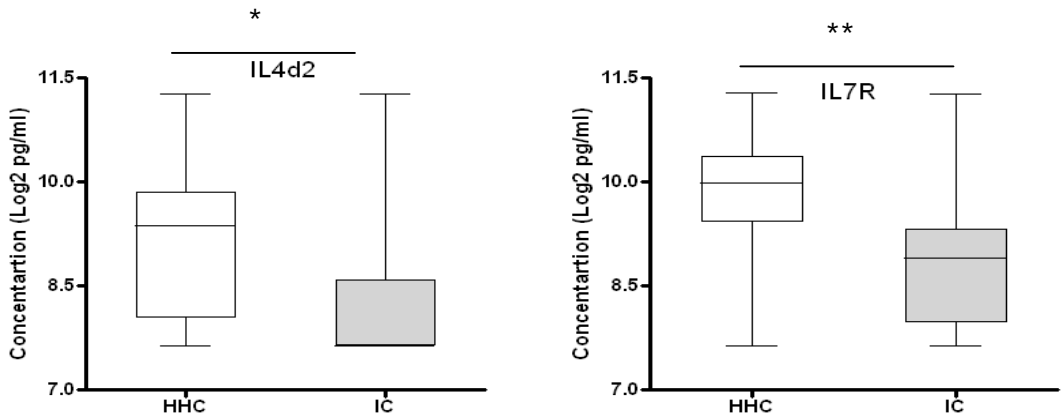


Figure 3-1 Gene expressions in household contacts and TB cases.
Box plots are shown where the horizontal lines indicate medians of household contacts (white bars) and TB cases (grey bars) and the lower and upper edge of each box indicate the 25th and 75th percentiles, respectively. Data were analysed using the non-parametric Mann-Whitney test with p-values indicating significant differences after transformation of Log2 values.*P<0.05;**P<0.001

We did a multivariate analysis and a combination of five genes gave a better discriminatory power with the combination of Bcl2, BLR1, MARCO FcgR1A and IL-4d2 detecting 95.65% of household contacts in a resubstitution classification matrix and after leave-one out cross validation. 95.83% of TB cases were correctly classified in a resubstitution classification matrix and after leave-one out cross validation (Table 3-2).

Table 3-1 General discriminate analysis of five marker combinations to discriminate active tuberculosis and household contacts

	Household contacts		TB cases				
Genes	Resubstitution clasification Matrix	Leave-one-out cross validation	Resubstitution classification Matrix	Leave-one-out cross validation	Wilks lambda value	f	p Value
Bcl2, BLR1, FcγR1A, IFN γ, IL4δ2	95.65	91.3	95.83	95.83	0.74	14	<0.001
Bcl2, FcγR1A, IFN γ, IL4δ2, MARCO	91.3	91.3	95.83	91.66	0.72	15.8	<0.001
Bcl2, BLR1, CD163, FcγR1A, IL4δ2	95.65	95.65	91.66	87.5	0.75	13.65	<0.001
Bcl2, BLR1,FcγR1A, IL4δ2, MARCO	95.65	95.65	95.83	91.66	0.73	14.95	<0.001
Bcl2, CD19,FcγR1A, IL4δ2, MARCO	95.65	95.65	91.66	91.66	0.75	13.21	<0.001
Bcl2, BLR1, CD19, FcγR1A, IL4δ2	91.3	91.3	95.83	87.5	0.77	11.98	0.0013
Bcl2, BPI, FcγR1A, IL4δ2, MARCO	95.65	95.65	91.66	95.83	0.72	16.11	<0.001
BLR 1, FcγR1A, IFN γ, IL4δ2, MMP9	95.65	86.95	95.83	91.66	0.75	13.46	<0.001
BLR 1, FcγR1A, IFN γ, IL4d2, RAB13	91.3	86.95	95.83	91.66	0.76	13.13	<0.001
Bcl2, FcγR1A, IL4δ2, MARCO, SPP1	95.65	95.65	95.83	87.5	0.71	16.7	<0.001

A general discriminant analysis (GDA) of the data indicated that optimal discrimination of TB cases and non-TB contacts could be achieved with combinations of 5 variables. FcγR1A and IL4δ2 were the most frequently occurring markers in the GDA biomarker combinations differentiating between the TB cases and household contacts (figure 3-2).

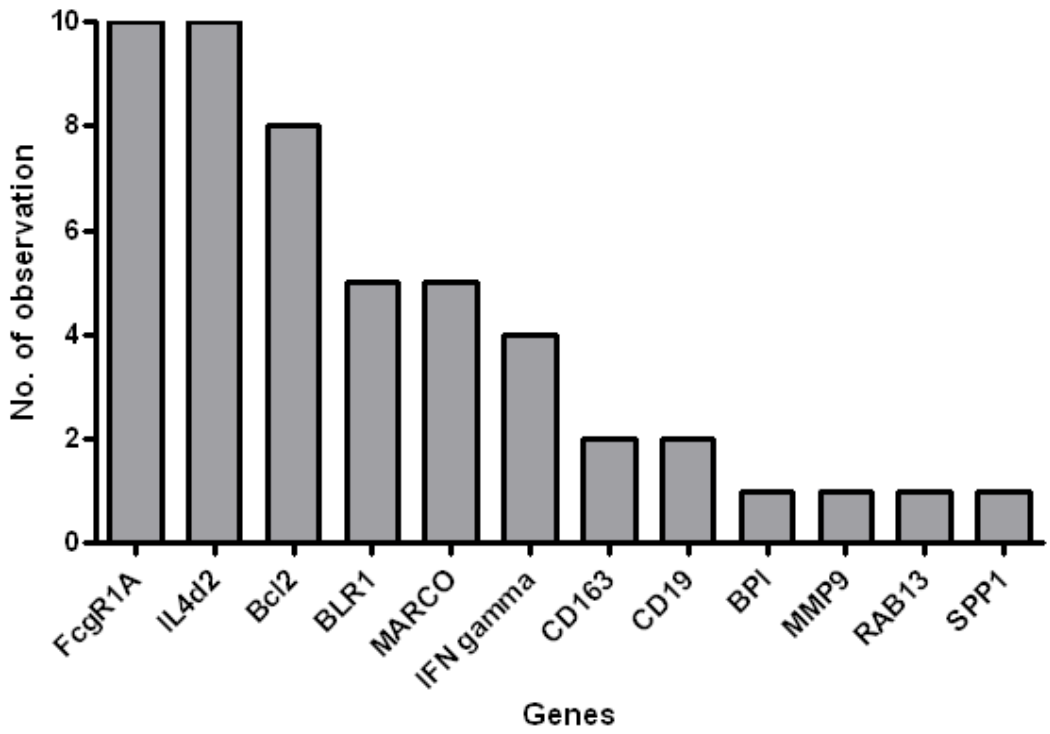
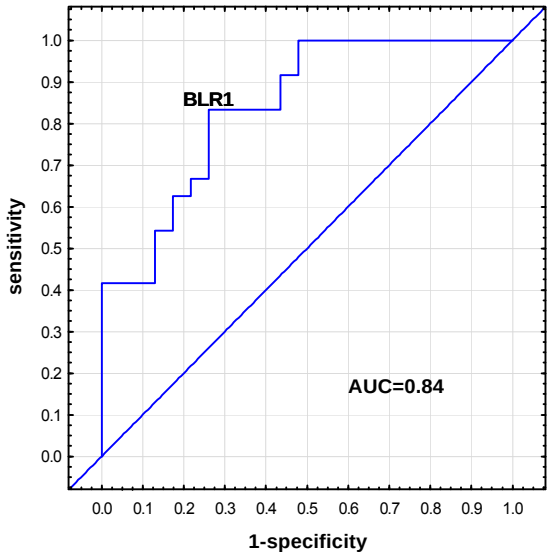
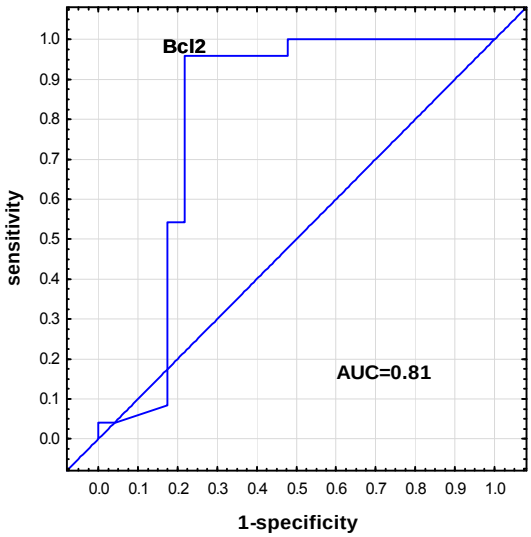
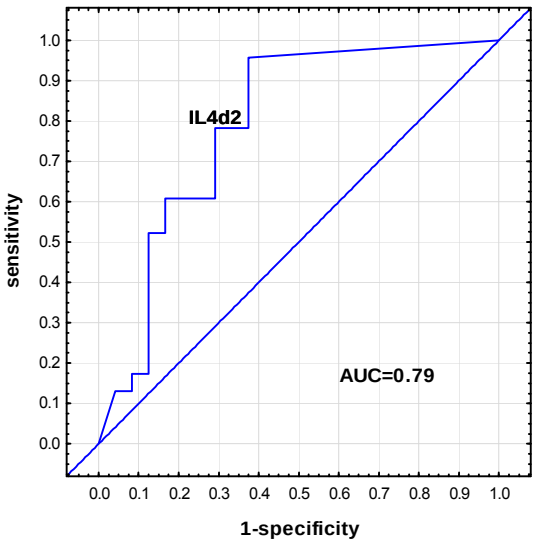


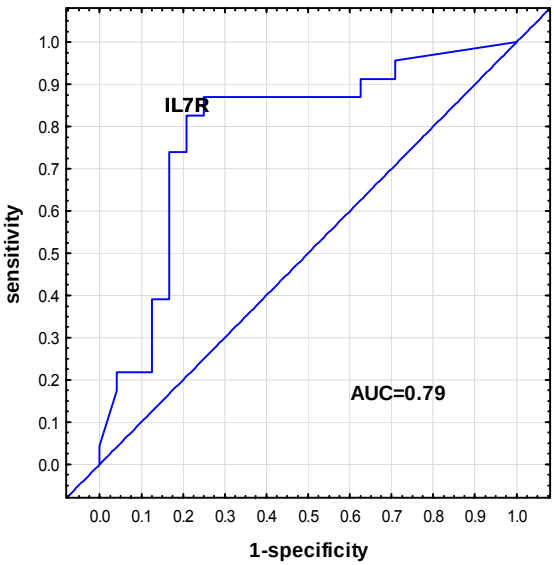
Figure 3-2 Frequency of individual analytes in top 10 models for discriminating between active TB cases and household contacts.
The columns represent the number of inclusions of individual markers into the most accurate five-analyte models by general discriminant analysis for discriminating between active pulmonary TB cases and contacts.

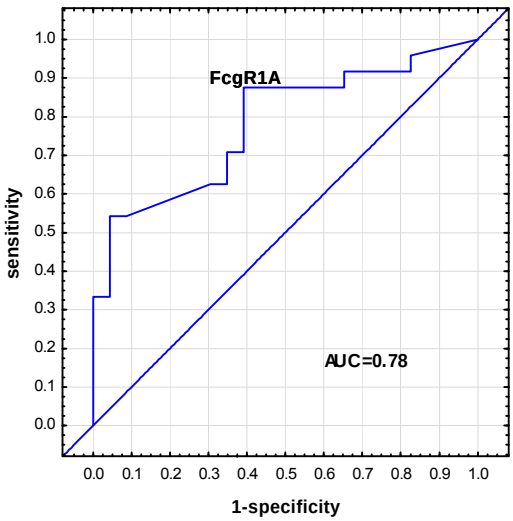
These most accurate single gene markers that differentiated TB cases and contacts were BLR1, Bcl2, IL-4d2, IL7R, FcgR1A, MARCO, MMP-9, CCL-19, and LTF with AUCs of 0.84, 0.81, 0.79, 0.79, 0.78, 0.76, 0.75, 0.75 and 0.68 respectively (Figure 3-3).











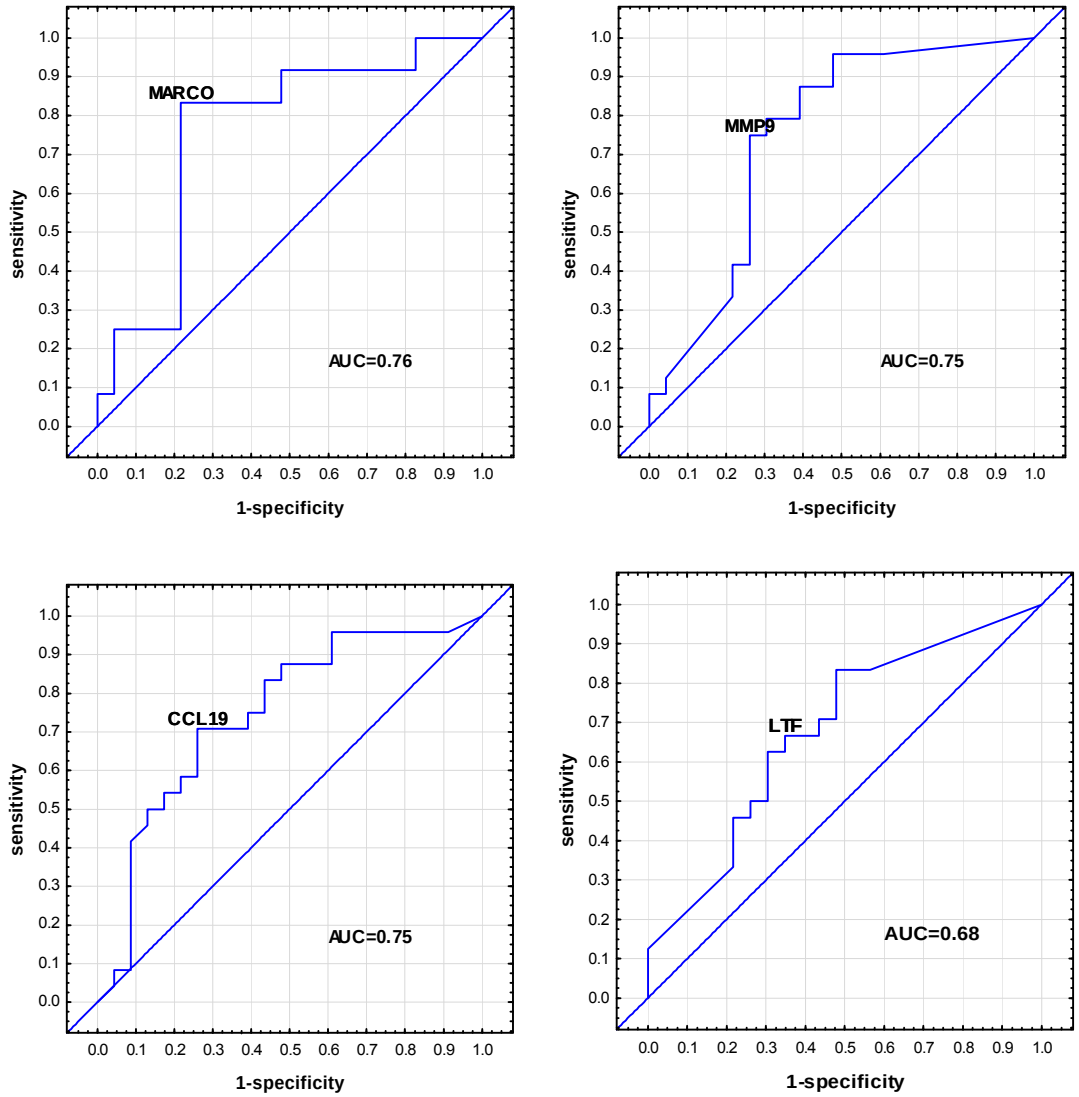


Figure 3-3 Receiver operator characteristics curves showing the accuracies of individual analytes in discriminating between active TB cases and household contacts.
Receiver operator characteristic (ROC) curves for the accuracies of single analytes to differentiate between active TB and household contacts. AUC = Area under the curve.

3.3.2 Gene expression of latently infected household contacts

We further classified the household contacts into latently infected and non-infected groups using the QFT-GIT test to see the effect of latent tuberculosis on the expression level of different genes. The expression level of Foxp3, CCL-19 and TGFβ were significantly higher ($p<0.05$) in QFT positive than QFT negative contacts (figure 3-4).

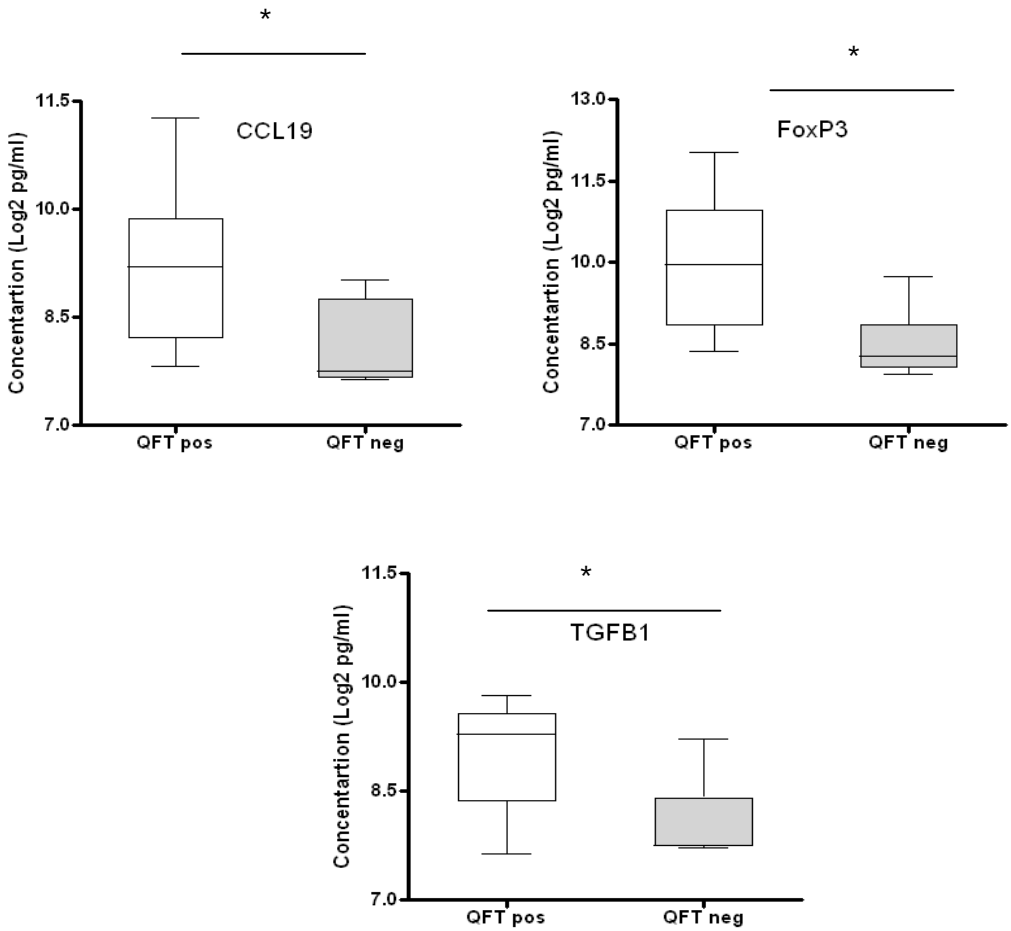


Figure 3-4 Gene expression in Quantiferon positive and Quantiferon negative household contacts.

Box plots are shown with the horizontal lines indicating median levels of Quantiferon positive (white bars) and Quantiferon negative (grey bars) household contacts. The lower and upper edge of each box indicates the 25th and 75th percentiles, respectively. Data were analysed using the nonparametric Mann-Whitney test with p-values indicating significant differences after transformation of data to Log2 values.*P<0.05.

We did multivariate analysis and found that a combination of five genes gave a better discriminatory level with the best performing combination of BPI, CCL19, FoxP3, FPR1 and TGFb1 detecting 90.9 % QFT negative household contacts in a resubstitution classification matrix. 90.9% of QFT negative contacts were correctly classified after leave-one out cross validation, 91.6% in a resubstitution classification matrix and 91.6% after leave-one out cross validation (Table 3-2).

Table 3-2 General discriminate analysis of five marker combinations to discriminate latently infected (QFT positive) and uninfected (Quantiferon negative) household contacts.

	QFT negative		QFT positive				
Genes	Resubstitution clasification Matrix	Leave-one-out cross validation	Resubstitution classification Matrix	Leave-one-out cross validation	Wilks lambda value	f	p Value
BPI, CASP8, CCL19, FOXP3 and TGFβ1	90.9	81.8	91.6	91.6	0.84	17.45	0.093
BPI, CCL19, FOXP3,TGFβ1 and TIMP2	90.9	81.8	83.3	83.3	0.74	17.67	0.027
CASP8, CCL13, CCL19, FOXP3 and TGFβ1	81.8	81.8	91.6	91.6	0.86	2.74	0.116
CCL19, CD14, FOXP3, IL2RA and TIMP2	90.9	90.9	83.3	83.3	0.62	10.22	0.005
CASP8, CCL19, FOXP3, RAB24 and TIMP2	81.8	81.8	91.6	91.6	0.92	1.54	0.23
CASP8, CCL19, CD163, FOXP3 and TGFβ1	90.9	81.8	91.6	91.6	0.92	1.53	0.23
CCL19, CD4, FOXP3, IL2RA and TIMP2	90.9	90.9	83.3	83.3	0.63	9.76	0.006
BPI, CCL19, FOXP3,FPR1 and TGFβ1	90.9	90.9	91.6	91.6	0.6	11.08	0.004
CASP8, CCL19, FASL, FOXP3 and TGFβ1	90.9	90.9	83.3	83.3	0.96	0.55	0.46
BPI, CCL19, FOXP3,SEC14L1 and TGFβ1	81.8	81.8	91.6	83.3	0.74	5.85	0.03

A general discriminant analysis (GDA) of the data indicated that optimal discrimination of latently infected and uninfected household contacts could be achieved with combinations of five variables, BPI, CCL19, Foxp3, FPR1 and TGFb1. A combination of BPI, CCL19, FoxP3, FPR1 and TGFb1 detects 90.9 % QFT negative household contacts in a resubstitution classification matrix and 90.9% after leave-one out cross validation and 91.6% detection of QFT negative in resubstitution classification matrix and 91.6% after leave-one out cross validation. FoxP3 and CCL19 were the most frequently occurring markers in the GDA biomarker combinations differentiating between latently infected and uninfected household contacts (Figure 3-5).

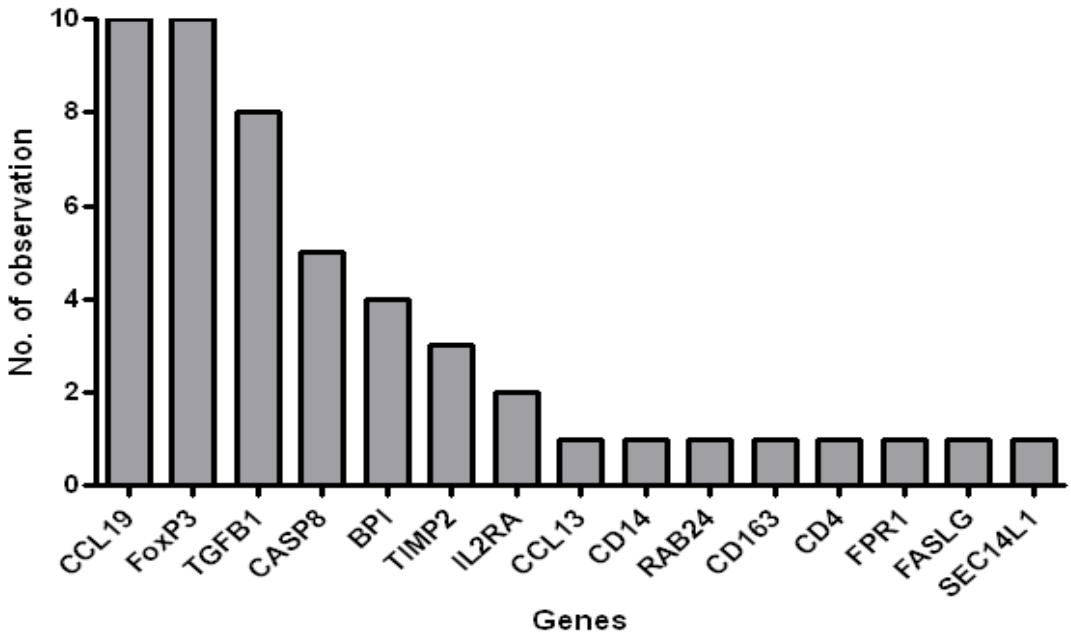


Figure 3-5 Frequency of individual analytes in top 10 models for discriminating between latently infected and uninfected household contacts.
The columns represent the number of inclusions of individual markers into the most accurate five-analyte models by general discriminant for discriminating between QFT positive and QFT negative contacts

The most accurate single gene markers that differentiated QFT positive and negative contacts were CCL-19, TGFb1 and Foxp3 with AUCs of 0.85, 0.82, and 0.75 respectively (Figure 3-6).

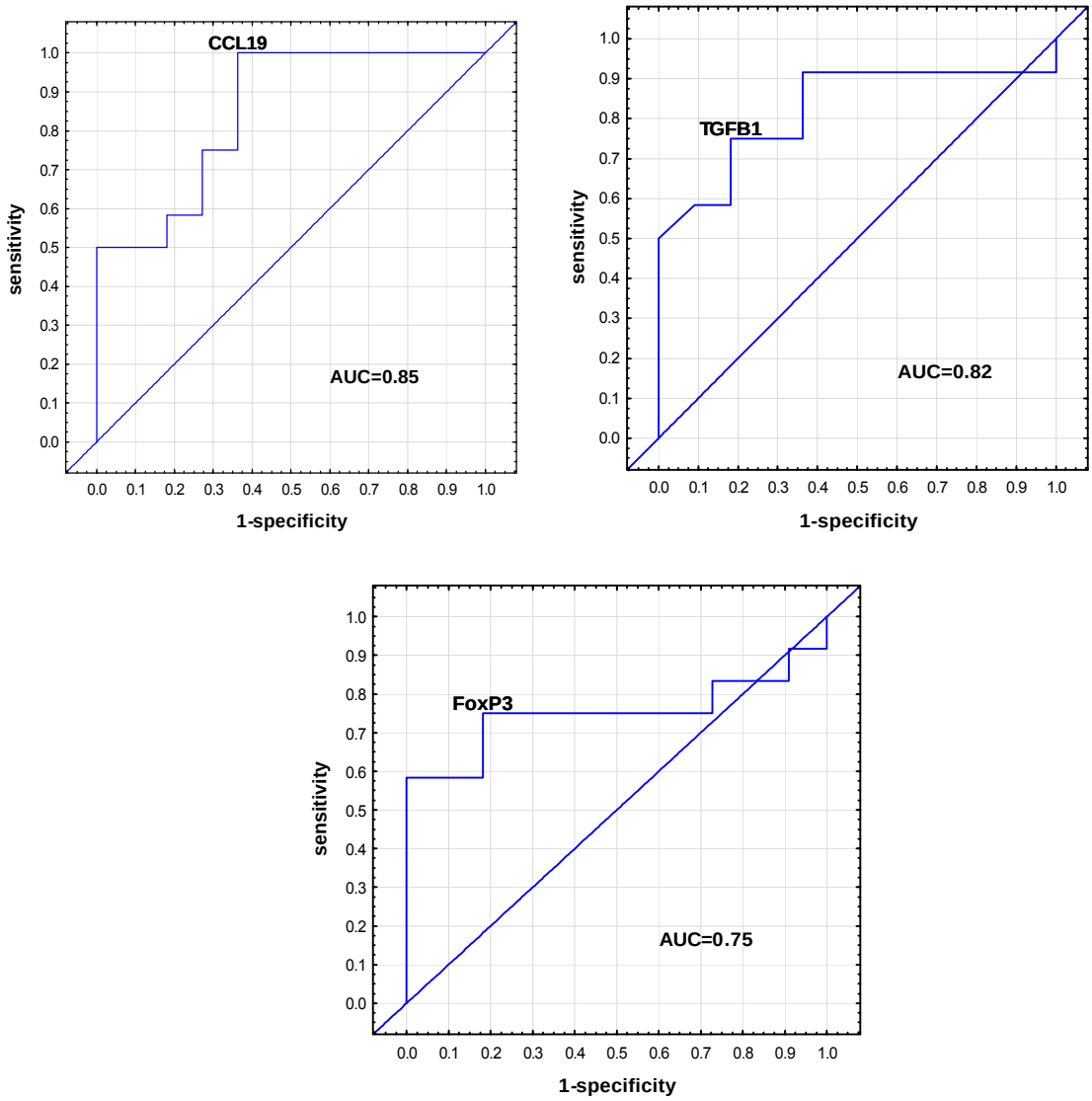


Figure 3-6 Receiver operator characteristics curves showing the accuracies of individual analytes in discriminating between latently infected and uninfected household contacts. Receiver operator characteristic (ROC) curves for the accuracies of single analytes to differentiate between latently infected and uninfected household contacts. AUC = Area under the curve.

3.3.3 Effect of HIV co-infection on gene expression of TB patients

We have also analysed the effect of HIV co-infection on the expression level of different genes in TB patients. We compared 12 patients in each group and we found that the expression levels of CD8a, LAG-3, IL-10, CCL-4 and CCL-22 were different in HIV positive and HIV negative TB patients. The level of CD8a, LAG-3, and IL-10 was significantly higher

(p<0.05) in HIV positive TB cases than HIV negative TB cases whereas the level of CCL-4 and CCL-22 was significantly higher (p<0.05) in HIV negative TB cases than HIV positive TB cases (figure 3-7).

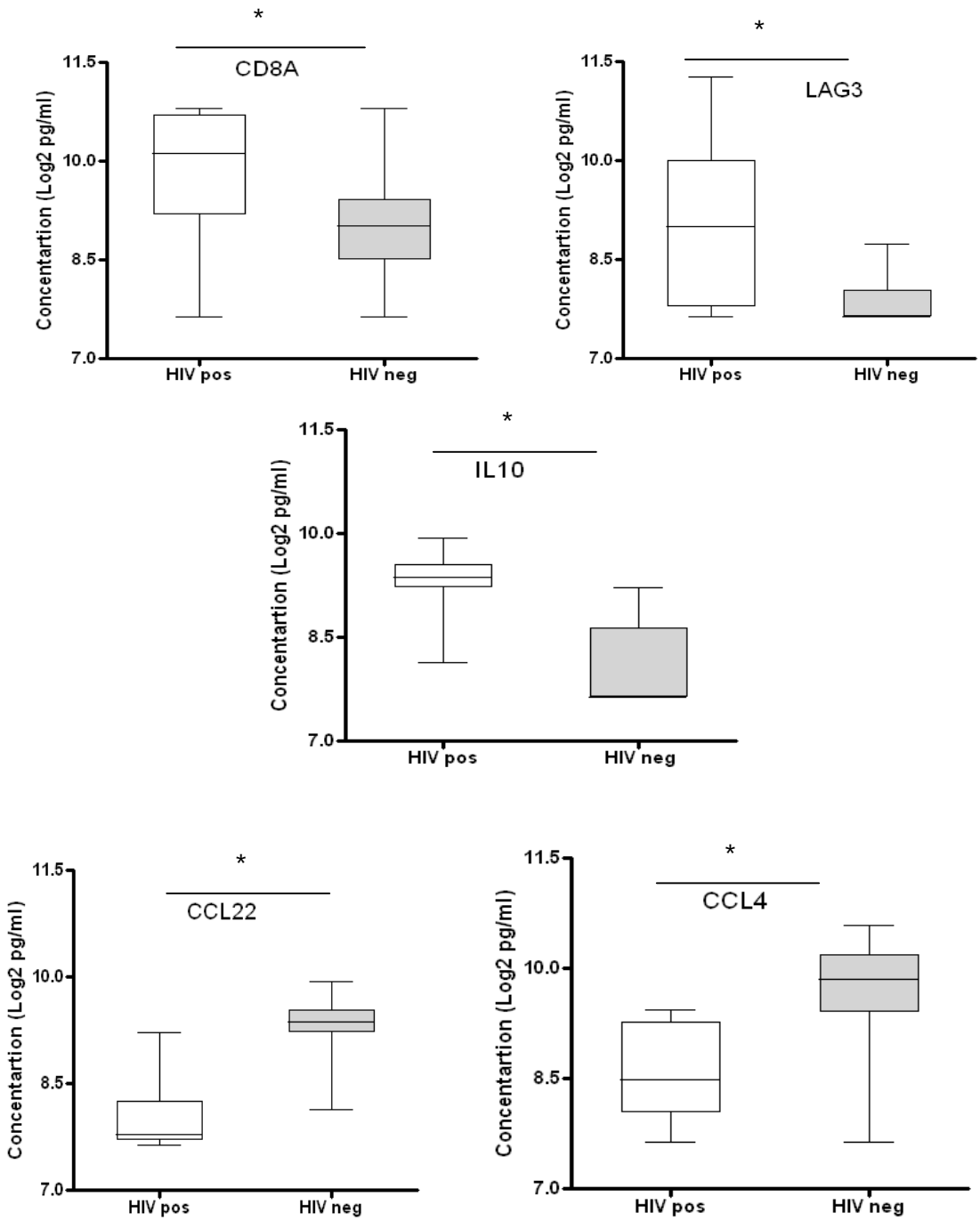


Figure 3-7 Gene expression in HIV positive and HIV negative TB cases.
Box plots are shown with the horizontal line indicating median expression of HIV positive (white bars) and HIV negative (grey bars) TB cases. Lower and upper edge of each box indicates the 25th and 75th percentiles, respectively. Data were analysed using the nonparametric Mann-Whitney test with p-values indicating significant differences after transformation of data to Log2 values.* P<0.05.

3.4 Discussion

Quantitative changes in gene expression levels can potentially be used as biomarkers to classify the different clinical groups of M. tuberculosis infection, as well as the effects of drugs and vaccines. We need a broader approach aimed at characterizing unique gene expression profiles due to the complexity of the infection process and outcomes of M. tuberculosis infection. A biomarker or combination of biomarkers that could be used to distinguish different clinical tuberculosis groups would be greatly beneficial.

In this study we used a dual colour reverse transcriptase MLPA technique to simultaneously identify multiple genes that are differentially expressed in tuberculosis cases and their contacts. We tested 45 genes and identified nine genes, which were differentially expressed between tuberculosis cases and their contacts.

The expression of FcgR1A, LTF, BLR-1, MARCO, CCL-19, MMP-9, CCL-4, and Bcl-2 in whole blood was statistically higher in TB patients than contacts, whereas the expression levels of IL-4d2 and IL-7R were statistically higher in healthy contacts. Similar to our finding the higher expression of FcgR1A and LTF in TB patients has been reported from previous studies in Germany (Jacobsen et al.; 2007) with microarray analysis of PBMCs from TB patients and healthy donors and in Gambia and Paraguay with MLPA (Joosten S et al.; 2012). FcgR1A and LTF are essential components of antimicrobial defense and blocking of induction of FcgR1A is one major target for the survival strategy of M. tuberculosis (Ting et al.; 1999). Lactoferrin modulates both the innate and adaptive immune response and there are reports showing the potential of lactoferrin as an adjuvant for BCG vaccination (Hwang et al.; 2011). Another study in a murine model also showed that susceptibility to TB could be reduced by avoiding overload of iron using LTF (Schaible et al.; 2002).

BLR-1 (CXCR5) is a chemokine receptor of a potent B cell attractor, CXCL-13, and the higher level of this gene in TB patients might help in sustaining the expression of its ligand CXCL-13 which in turn attracts B cells. The role of B cells in immune response against TB has been documented in various studies (Abebe and Bjune; 2009; Zhang et al.; 2011). The higher level of CCL-19 in TB patients could be due to the presence of active infection where a number of important cells including macrophages and T cells are recruited for containing infection. Different in vivo and in vitro studies indicated that M. tuberculosis infection of human monocyte derived macrophages, alveolar macrophages, and CD4 T cells induced upregulation of chemokine receptors and their ligands (Kurashima et al.; 1997; Lin et al.; 1998; Algood et al.; 2003; Pokkali and Das; 2009).

The higher level of MMP-9 and MARCO in TB infections is in line with other previous reports, which reported a higher level of MMP-9 in TB cases where it facilitates early dissemination of the bacteria with subsequent recruitment of macrophages, induction of Th1 type immunity and granuloma formation (Izzo et al.; 2004; Taylor et al.; 2006). MARCO is a phagocytic receptor and M. tuberculosis uses different receptors to infect macrophages. Previous work in mouse models also showed upregulation of MARCO genes after BCG infection (Ito et al.; 1999) and a low proinflammatory response of $MARCO^{-/-}$ mice in response to infection with virulent M. tuberculosis (Bowdish et al.; 2009).

The other genes, which had a discriminatory expression, were Bcl-2 and IL-4d2. Bcl-2 is an anti-apoptotic gene and in this study its expression was higher in TB patients. Apoptosis is believed to be one mechanism of immune responses by the host for eliminating infected cells without releasing viable bacteria and previous studies in Ethiopia and Gambia (Abebe et al.; 2010; Joosten S et al.; 2012) indicated upregulation of apoptotic genes in TB patients

although we could not repeat their finding in this study. However, the higher expression of Bcl-2 in this study could be a survival strategy of the pathogen as previous studies also show that M. tuberculosis or its derived products inhibited apoptosis (Abebe et al.; 2011). Expression of IL-4d2 is higher in contacts and it is a recently described splice variant of IL-4, which inhibits IL-4 activity. Different studies showed that latently infected individuals express high levels of Th1 cytokines and the IL-4 antagonist IL-4d2 and individuals with a high IL-4δ2/IL-4 ratio controlled M. tuberculosis infection (Wassie et al.; 2008).

The expression of CCL-19, TGFb1, and Foxp3 significantly discriminates latently infected and uninfected contacts. The expression of all genes was higher in latently infected individuals and their higher expression might be due to a recent M. tuberculosis infection, which could lead to immune activation and recruitment of immune cells. CCL-19 is an important molecule for recruiting activated immune cells and increased expression of regulatory molecules could be important to avoid prolonged immune activation and immunopathology as regulatory molecules like Foxp3, and TGFβ1 regulate immune response during infection to prevent excessive inflammation and tissue damage (Ribeiro-Rodrigues et al.; 2006). Another study also showed activation and expansion of both T effector cells and Foxp3 (+) Treg populations early in M. tuberculosis infection. IL-2 induces expression of both effector Tcells and Foxp3 and confers resistance against severe M. tuberculosis infection (Chen et al.; 2012).

The other groups, which showed a discriminatory gene expression profile, were HIV positive and HIV negative TB patients. The expression of CD8a, LAG-3, IL-10, CCL-4 and CCL-22 significantly discriminates the two groups where CD8a, LAG-3 and IL-10 genes were higher in HIV positive TB cases than in HIV negative TB cases whereas the expression level of CCL-4

and CCL-22 was higher in HIV negative TB patients. The reduced level of CCL-4 and CCL-22 in HIV positive TB cases could be due to the significant loss of CD4 cells due to HIV. In one in vitro study it was shown that activated CD4 cells produce HIV 1 suppressive factors, like CCL-22 and CCL-4. These act on CCR-4 and CXCR-5 receptors (Abdelwahab et al.; 2003). The higher level of IL-10 in co-infected TB patients might be due to the immune exhaustion induced by HIV where the HIV-specific CD4 T cell response is inhibited through upregulation of IL-10. Previous studies suggested that the level of IL-10 is elevated in chronic HIV disease (Kwon D et al.; 2012). The higher level of CD8a in HIV positive TB patients could be due to the role of CD8 cells in limiting disease progression during chronic infection as there are studies which showed very rapid HIV/AIDS progression in the absence of HIV-1-specific CD8+ T cells (Feng et al.; 2010).

In conclusion, active tuberculosis cases versus non tuberculosis cases or latently infected versus uninfected contacts could be accurately differentiated using expression of single genes with combinations of genes providing improved discriminatory power in differentiating between different clinical groups of tuberculosis infection. However, there is a need to validate gene biosignatures in larger studies and in other cohorts prospectively.

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CHAPTER 4

4. Plasma cytokines and chemokines differentiate between active and non-active tuberculosis infection

Abstract

Background: Identifying biomarkers that could differentiate between the different clinical conditions of M. tuberculosis infection and treatment outcomes have paramount importance in TB control.

Method: We analysed ex vivo plasma samples from 33 TB patients (17 HIV negative and 16 HIV positive) and 30 healthy household contacts with Luminex.

Result: We found statistically significant differences ($p<0.05$) in median plasma levels of EGF, fractalkine, IFN-γ, IL-4, MCP-3 and IP-10 between contacts and TB patients. However, none of the cytokines were significantly different in latently infected and uninfected contacts ($P>0.05$). HIV does not affect the median plasma level of any of the cytokines or chemokines and there was not significant difference between HIV positive and HIV negative TB patients ($P>0.05$) in any of the cytokines or chemokines. The median plasma levels of IFN-γ, IL-4, MCP-3, MIP-1β and IP-10 were significantly different ($P<0.05$) before treatment and after treatment.

Conclusion: Plasma cytokines and chemokines could be used as immunological markers for diagnosing active TB disease and for monitoring effective anti tuberculosis therapy.

4.1 Introduction

A more simple and accurate diagnosis of active TB disease and latent tuberculosis and monitoring of anti-TB treatment are crucial for curbing the current TB epidemic. The more than a century old smear microscopy test is the most widely used technique for TB diagnosis and for monitoring efficacy of anti TB therapy. Similarly, until recently the old tuberculin skin test (TST) has been used solely as a diagnostic test for latent tuberculosis infection. However, in recent times two new in vitro T cell based immuno assays were developed, which measure IFN gamma (IFN-γ) response against M. tuberculosis specific antigens, early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10). The two T-cell-based Interferon Gamma Release Assays (IGRAs) are commercially available and widely used for diagnostic and research purposes: QuantiFERON TB-GOLD In-Tube (QFT-G-IT) (Cellestis Ltd., Carnegie, Australia) which includes an additional M. tuberculosis-specific antigen TB7.7 and T-SPOT.TB (Oxford Immunotec, Abingdon, UK). These tests are reported to be less affected by Bacillus Calmette Guerin (BCG) vaccination than the TST and different studies indicated that IGRAs are more sensitive and specific than TST (Diel et al.; 2010). However both tests do not differentiate active TB from latent TB infection.

There are different studies using different approaches and samples reporting different cytokines and or chemokines with a potential of discriminating the different clinical outcomes of M. tuberculosis infection and anti TB treatment. Stimulation of PBMC with M. tuberculosis antigens gives rise to high levels of Th1 cytokines and the IL-4 antagonist IL-4d2 in protected individuals in comparison with non protected individuals (Demissie et al.; 2004) and proinflamatory cytokines such as TNF, IL-12(p40) and IL-17 are increased in TB cases and can discriminate between active TB disease and latent infection (Sutherland et al.;

2010). Serum or plasma of TB cases have high levels of IL-8, IP-10, MCP-1, and MIP-1 β in comparison with non TB cases (Juffermans et al.; 1999; Azzurri et al.; 2005; Siawaya et al.; 2009a) and mRNA level of individuals with a high IL-4δ2/IL-4 ratio controlled M. tuberculosis infection (Wassie et al.; 2008). Another study also showed that levels of single or combinations of three host markers (selected from EGF, sCD40L, MIP-1•, VEGF, TGF-• or IL-1•) in stimulated or unstimulated plasma samples from QFT supernatants had promising discriminating ability between latent and active TB (Chegou et al.; 2009). The increase of some chemokines in supernatants from whole blood stimulated with Mycobacterium tuberculosis-specific antigens was reported to be a marker of tuberculosis infection (Ruhwald et al., 2011).

On the basis of this line of research, in the current study we addressed the possibility of using cytokines and chemokines from unstimulated plasma samples for detection of active TB disease, latent TB, discriminating active TB cases from latently infected contacts and for monitoring anti TB treatment outcome.

4.2 Materials and methods

Study subjects

In this prospective study, 33 subjects with active TB disease (17 HIV negative and 16 HIV positive) and 30 healthy household contacts (15 QFT-GIT positive and 15 QFT-GIT negative) were recruited from selected health centers in Addis Ababa, Ethiopia. The diagnosis of TB in the health centres was based on the national guidelines of at least two positive smears for acid-fast bacilli (AFB) in three specimens collected from each patient as spot-morning-spot. All sputum samples from TB cases were cultured for mycobacteria following the protocol described in the methodology section (2.4.6).

Household contacts were recruited through the active TB cases following the definition in the methodology section. The presence of HIV infection was ruled out using rapid tests (Stat pack, KHP and Unigold as a tie breaker) as per the national guideline. QFT-G-IT test was used to detect latent tuberculosis and we measured plasma cytokines using Luminex as described in the methodology part of the thesis.

Statistical analysis

The data were analyzed using Graph pad prism software, version 4.0. Nonparametric Mann–Whitney U tests were performed for comparison between groups A multivariate analysis was done using STATISTICA Version 10. A P value less than 0.05 was considered as statistically significant.

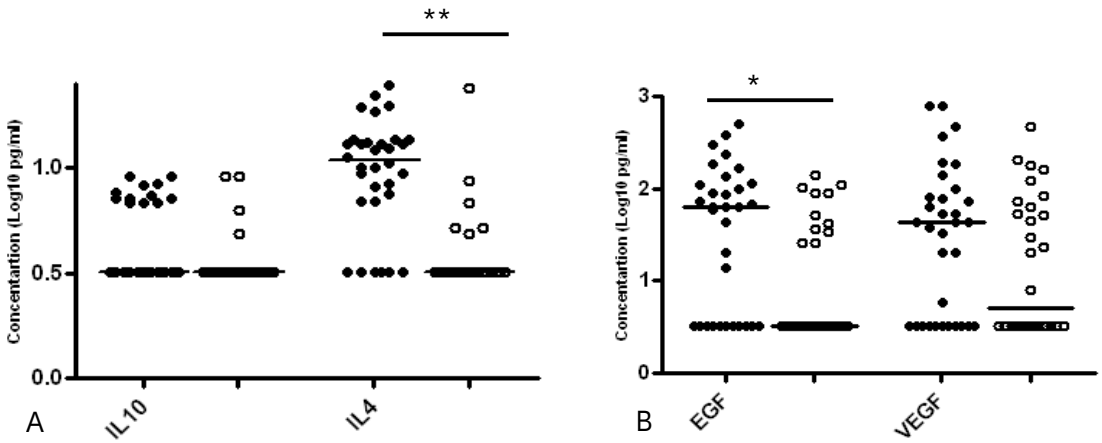
4.3 Result

4.3.1 Demographic characteristics

We enrolled 33 subjects with active tuberculosis and 30 household contacts. Among the TB cases, 16 were HIV positive and all were ART naïve with a mean CD4 count of 310 ±23.9 cells/•L. The household contacts comprising 15 QFT-GIT positive and 15 QFT-GIT negative contacts were all HIV negative. The mean age of TB patients was 32.4 ± 1.7 and 48.5% of the participants were females. The mean age of household contacts was 29.7±2.7 and 47.4% of household contacts were female.

4.3.2 Cytokines and /or chemokines diagnostic potential of TB cases

Plasma samples from 33 TB patients (17 HIV negative and 16 HIV positive) and 30 healthy household contacts were analysed with Luminex without any stimulation and we found that significantly different levels of cytokines and/or chemokines between the different groups. The median level of EGF, FRACTALKINE, IFN-γ, IL-4, MCP-3 and IP-10 were statistically significant different ($p<0.05$) between TB patients and their contacts (Figure 4-1).



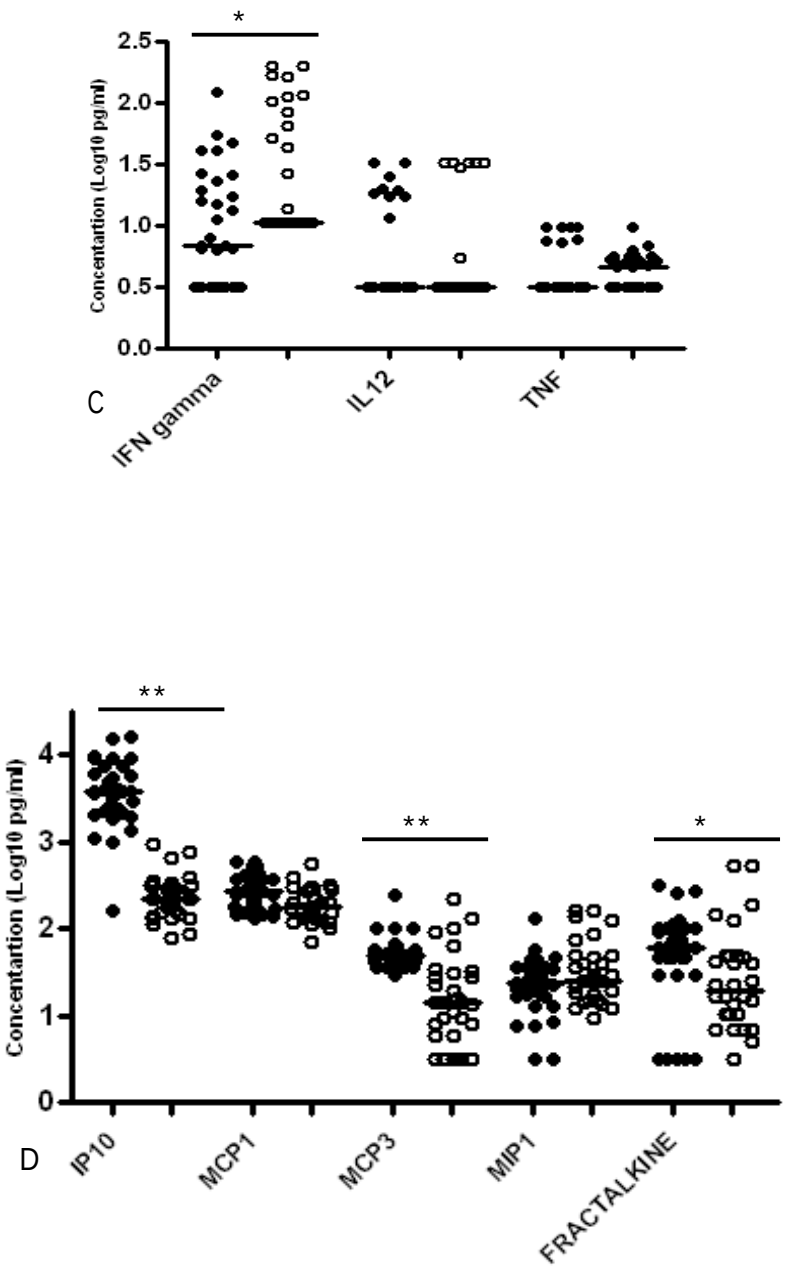


Figure 4-1 Plasma cytokine and chemokine levels in TB cases and their household contacts.

Unstimulated plasma samples from TB cases (n = 33) and household contacts (n = 30) were assessed by multiplex cytokine analysis. A) Anti-inflammatory cytokines (IL-10 and IL-4), B) Growth factors (EGF and VEGF), C) Pro-inflammatory cytokines (IFN-γ, IL 12(p40), and TNF) and D) Chemokines (IP-10, MCP-1, MCP-3, MIP-1β and fractalkine). The horizontal line indicates median values of TB cases (filled circles) and household contacts (open circles). Data were analysed using nonparametric Mann-Whitney test with p-values indicating significant differences after transformation of data to Log10 values.*$p<0.05$, **$p<0.001$.

The list below shows median levels and ranges of all markers that were statistically significantly different between cases and contacts. The levels of markers in contacts were: EGF (0.5pg/ml, range 0.5-2.1pg/ml), fractalkine (0.5pg/ml, range 0.5-2.7pg/ml), IFN-γ (1.03pg/ml, range 1.03-2.2pg/ml), IL-4 (0.5pg/ml, range 0.5-1.7pg/ml), MCP-3 (1.16pg/ml, range 0.5-2.3pg/ml) and IP-10 (2.34pg/ml, range 1.9-2.98pg/ml). Median levels and ranges in TB patients were: EGF (1.79pg/ml, range 0.5-2.7pg/ml), fractalkine (1.79pg/ml, range 0.5-2.5pg/ml), IFN-γ (0.84pg/ml, range 0.5-2.09pg/ml), IL-4 (1.04pg/ml, range 0.5-1.39pg/ml), MCP-3 (1.68pg/ml, range 1.03-2.2pg/ml), IP-10 (3.57pg/ml, range 2.2-4.2pg/ml).

We did multivariate analysis and found that a combination of five cytokines gave a better discriminatory level with the best model including a combination of fractalkine, IFN-γ, IL-4, IP-10 and TNF with 96.87 % detection of TB cases in a resubstitution classification matrix and with 96.7% after leave-one out cross validation and 100% detection of household contacts (Table 4-1).

Table 4-1 General discriminate analysis of five marker combinations to discriminate active tuberculosis and household contacts

	TB cases		Non TB cases				
Cytokines	Resubstitution clasification Matrix	Leave-one-out cross validation	Resubstitution classification Matrix	Leave-one-out cross validation	Wilks lambda value	f	p Value
fractalikine, IFN g, IL 4, IP 10 and TNF	96.87	96.87	100	100	0.69	25	<0.0001
fractalkine, IFN g, IP 10, MCP 3 and MCP 1	96.87	93.75	100	93.3	0.81	13	0.0006
fractalkine, IFN g, IL 4, IP 10 and MIP 1	96.87	93.75	100	100	0.69	24	<0.0001
IFN G, IL 12, IP 10, MCP 3, and MIP 1	96.87	93.75	96.66	93.33	0.85	9.4	0.003
IFN G, IL 12, IP 10, MCP 3, and TNF	96.87	96.87	96.66	93.33	0.77	16	<0.0001
fractalkine, IFN g, IL 12, IL4 and IP 10	96.87	96.87	100	100	0.54	48	<0.0001
EGF, fractalkine, IFN g, IL 4 and IP 10	96.87	93.75	100	100	0.51	53	<0.0001
fractalkine, IFN g, IP 10, MCP 3, and TNF	96.96	93.93	96.66	93.33	0.7	24	<0.0001
fractalkine, IFN g, IL 12, IP 10, and MCP 3	96.87	96.87	96.66	93.33	0.57	42	<0.001
fractalkine, IFN g, IL 4, IP 10 and VEGF	96.87	96.87	100	100	0.51	53	<0.001
IFN G, IP 10, MCP 3, MIP 1 and TNF	96.87	96.87	96.66	93.33	0.9	5.9	0.018
fractalkine, IFN g, IL 4, IP 10 and MCP 1	96.87	96.87	100	96.66	0.74	20	<0.0001
fractalkine, IFN g, IL 12, IP 10 and TNF	96.87	93.75	100	96.66	0.7	23	<0.0001
EGF, fractalkine, IFN g, IL 4, IP 10 and TNF	93.75	93.75	100	100	0.69	25	<0.0001
IFN g, IL 4, IP 10, MCP 3 and TNF	96.87	96.87	96.66	93.33	0.75	19	<0.0001
fractalkine, IFN g, IP 10, MCP 1, and TNF	96.96	93.93	100	96.66	0.8	14	0.0004
fractalkine, IFN g, IP 10, MIP 1, and TNF	93.75	93.75	100	96.66	0.77	17	0.00014
fractalkine, IFN g, IL 10, IL4 and IP 10	96.87	96.87	100	100	0.54	47	<0.0001
fractalkine, IFN g, IL 4, IP 10 and MCP 3	96.87	93.75	100	93.33	0.52	51	<0.0001
EGF, IFN g, IP10, MCP 3, MIP 1	93.75	93.75	93.33	93.33	0.84	11	0.0019

A general discriminant analysis (GDA) of the data indicated that optimal prediction of TB cases could be achieved with combinations of 5 variables. IP-10 and IFN-γ were the most frequently occurring markers in the GDA biomarker combinations differentiating between the TB cases and household contacts (figure 4-2).

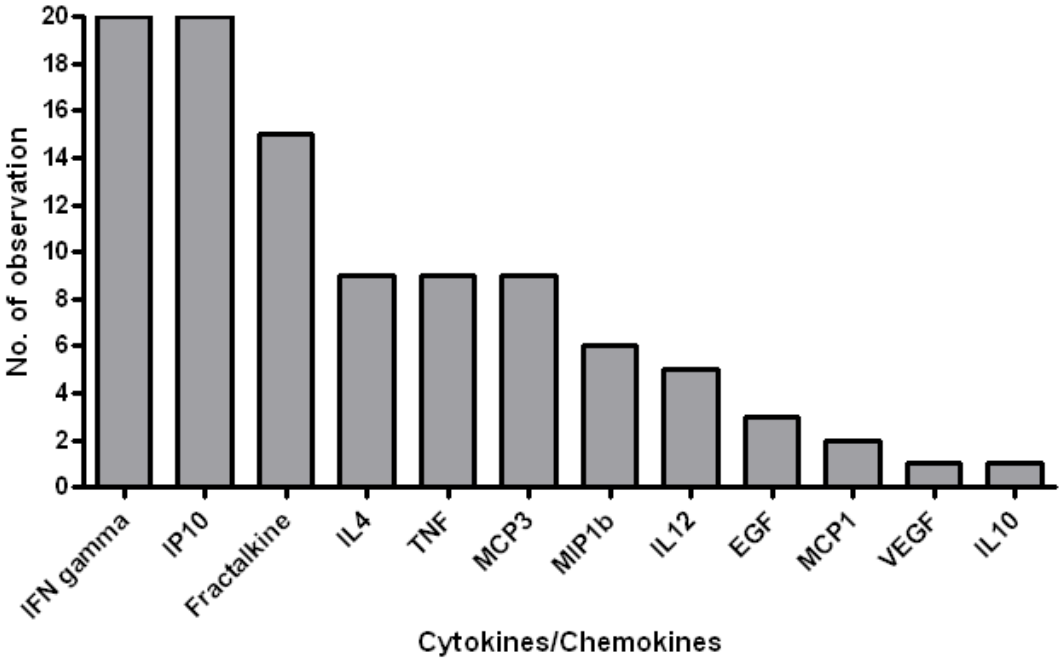


Figure 4-2 Frequency of individual analytes in top 20 models for discriminating between active TB cases and household contacts.

The columns represent the number of inclusions of individual markers into the most accurate five-analyte models by general discriminant for discriminating between active pulmonary TB cases contacts.

4.3.3 Diagnostic potential of cytokines and /or chemokines in latent TB cases

Moreover, we further classified the household contacts into latently infected and non-infected groups by using Quantiferon-Gold in tube test. Similarly, the plasma level EGF, FRACTALKINE, IFN-γ, IL-4, MCP-3 and IP-10 were statistically higher in TB patients than QFT positive or QFT negative subjects ($p<0.05$), however, none of the cytokines were able to differentiate QFT positive from QFT negative groups ($p>0.05$) (figure 4-3).

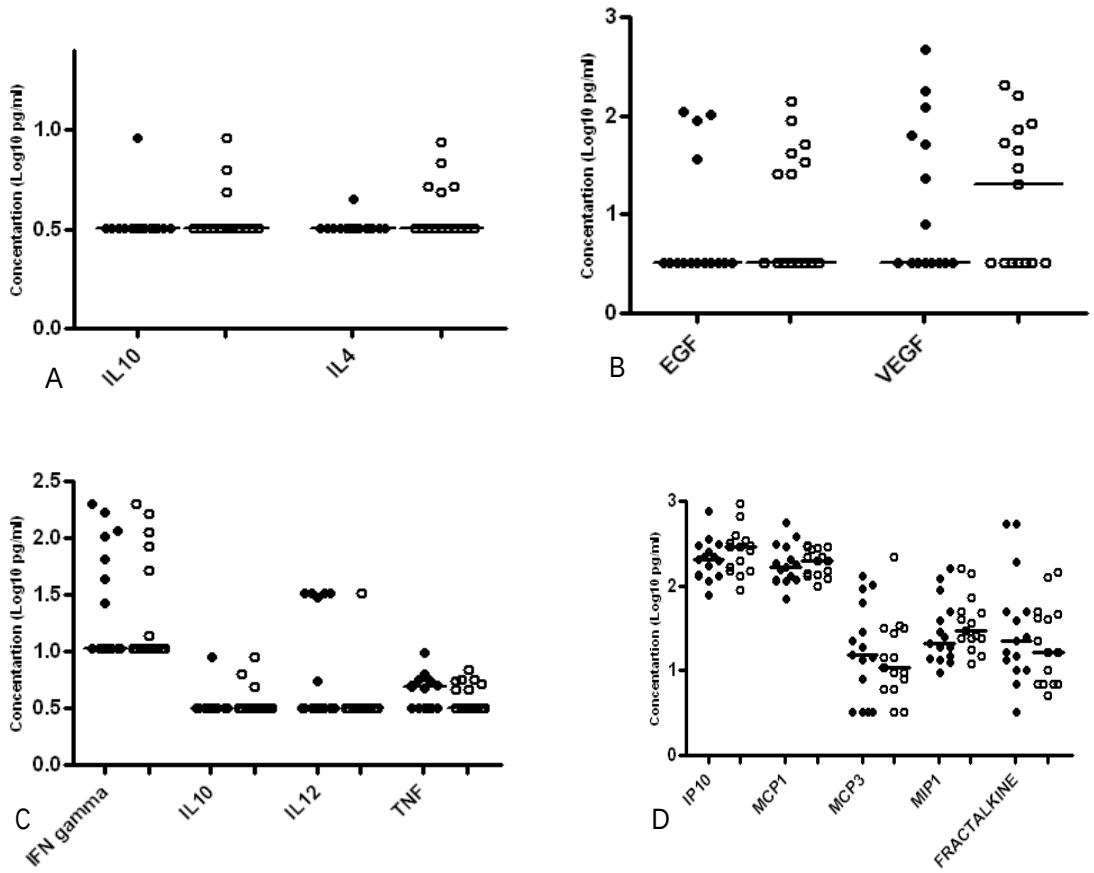


Figure 4-3 Plasma cytokine and chemokine levels in QFT negative and QFT positive household contacts.

Unstimulated plasma samples from QFT negative (n = 15) and QFT positive (n = 15) were assessed by multiplex cytokine analysis. A) Anti-inflammatory cytokines (IL-10 and IL-4), B) Growth factors (EGF and VEGF), C) Pro-inflammatory cytokines (IFN-γ, IL 12(p40), and TNF) and D) Chemokines (IP-10, MCP-1, MCP-3, MIP-1β and fractalkine). Horizontal line indicates median of QFT negative (filled circles) and QFT positive (open circles). Data were analysed using nonparametric Mann-Whitney test with p-values indicating significant differences after transformation of data to Log10 values.

4.3.4 Diagnostic potential of cytokines and /or chemokines in HIV positive and HIV negative TB cases

We found that the median plasma level of any of the cytokines/chemokines was not affected by HIV infection status although the HIV positive TB patients have a slightly higher level of most of the cytokines and chemokines (Fig 4-4).

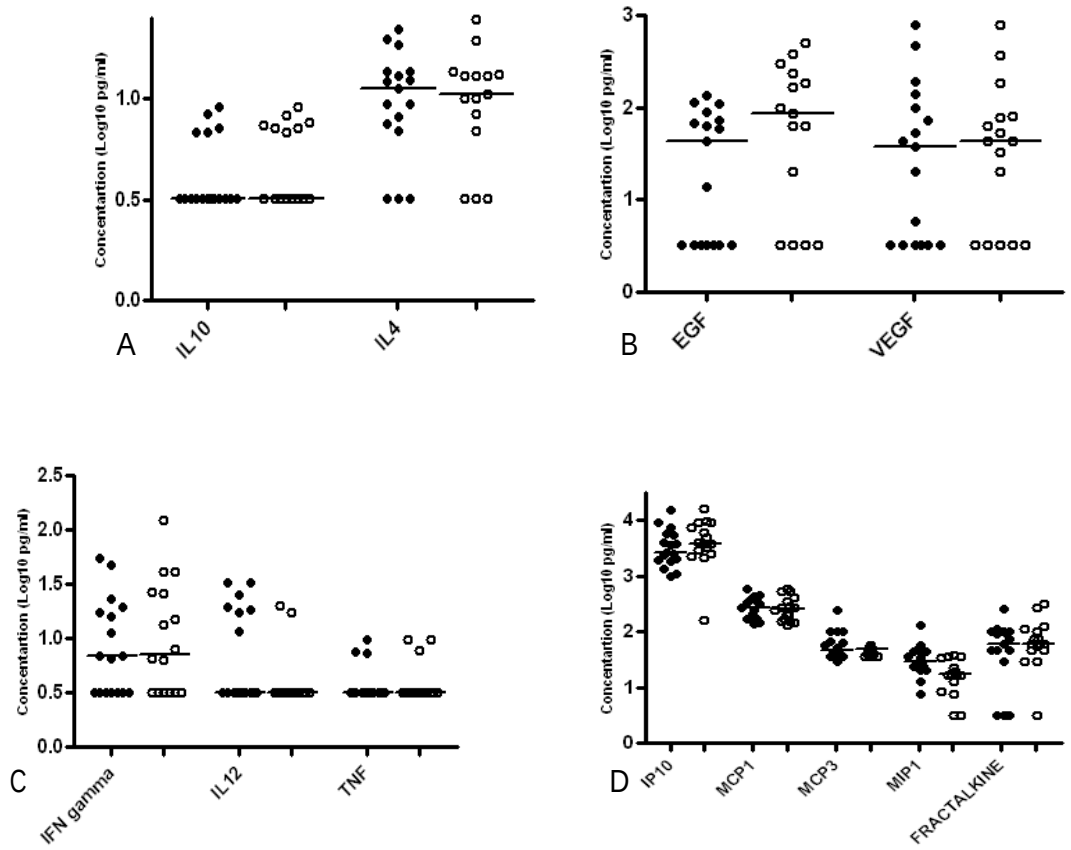
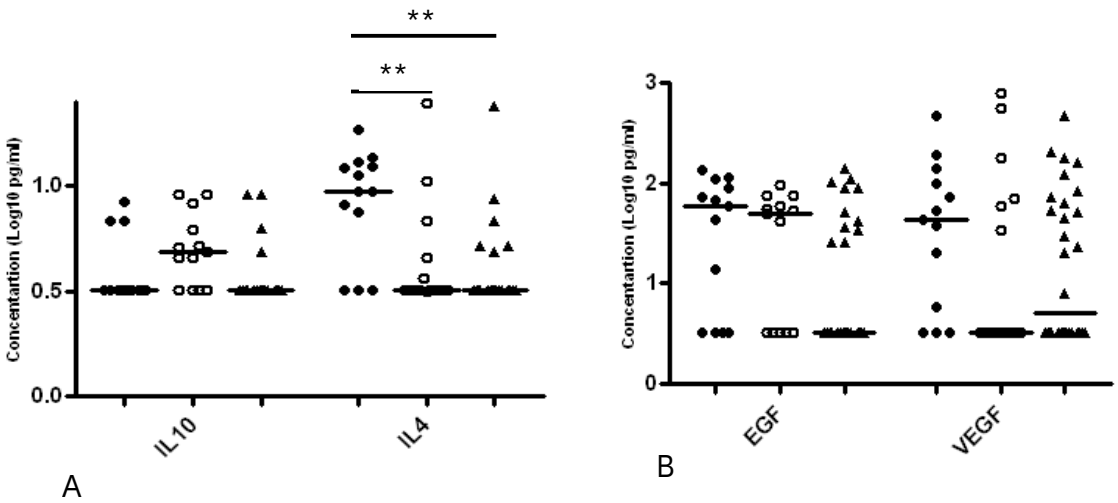


Figure 4-4 Plasma cytokine and chemokine level in HIV negative and HIV positive TB cases.

Unstimulated plasma samples from HIV positive TB cases (n = 16) and HIV negative TB cases (n = 17) were assessed by multiplex cytokine analysis. A) Anti-inflammatory cytokines (IL-10 and IL-4), B) Growth factors (EGF and VEGF) C), Pro-inflammatory cytokines (IFN-γ, IL 12(p40), and TNF) and D) Chemokines (IP-10, MCP-1, MCP-3, MIP-1β and fractalkine). Horizontal lines indicate median of HIV negative (filled circles) and HIV positive TB cases (open circles). Data were analysed using nonparametric Mann-Whitney test with p-values indicating significant differences after transformation of data to Log10 values.

4.3.5 Cytokines and /or chemokines levels in TB cases before and after treatment

We also measured the plasma level of the cytokines and chemokines after 8 month of effective anti-TB therapy to see if any of these cytokines and/or chemokines showed a treatment effect and we found that the median level of IFN-γ, IL-4, IP-10, MCP-3 and MIP-1β were statistically different ($p<0.05$) before treatment and after treatment (figure 4-5). All the cytokines that differentiated between TB cases and controls at baseline either increased or decreased to the level of household contacts after treatment, except MIP 1β were its level was not decreased to the level of household contacts.



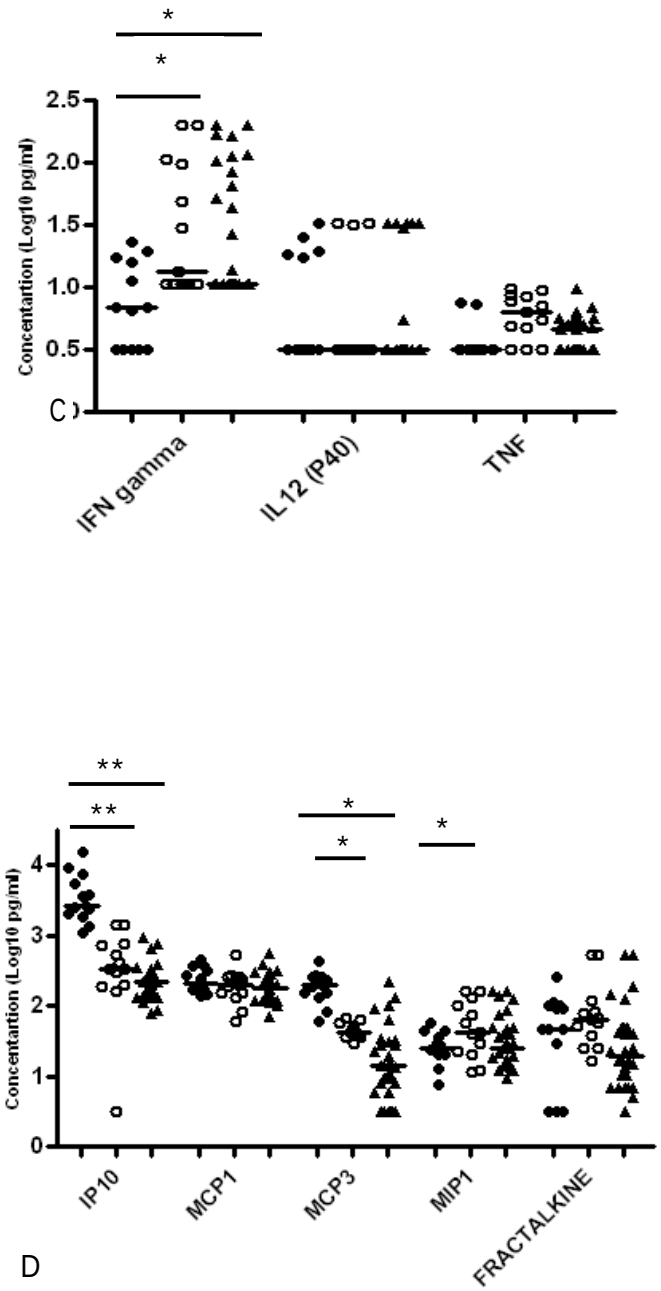


Figure 4-5 Plasma cytokine and chemokine levels in TB cases before treatment, after treatment and in household contacts.

Unstimulated plasma samples from TB cases before treatment (n = 15), TB cases after treatment (n=15) and household contacts (n = 30) was assessed by multiplex cytokine analysis. A) Anti-inflammatory cytokines (IL-10 and IL-4), B) Growth factors (EGF and VEGF), C) Pro-inflammatory cytokines (IFN-γ, IL 12(p40), and TNF) and D) Chemokines (IP-10, MCP-1, MCP-3, MIP-1β and fractalkine). Horizontal line indicates median of TB cases before treatment (filled circles), TB cases after treatment (open circles) and household contacts (filled triangle). Data were analysed using nonparametric Mann-Whitney test with p-values indicating significant differences after transformation of data to Log10 values. *p<0.05, **p<0.001.

4.4 Discussion

In this study we determined the levels of different cytokines and chemokines directly from plasma in an endemic population as part of an effort to identify a single marker or combination of cytokines and/or chemokines which have a potential to detect active TB, differences between pre- and post-TB treatment time points and to discriminate between infected and uninfected individuals. The main findings of this study are: (a) TB patients had significantly higher plasma concentrations of EGF, fractalkine, IL-4, MCP-3 and IP-10 and lower plasma concentration of IFN-γ compared to household contacts irrespective of QFT status and; (b) None of the cytokines or chemokines measured were able to discriminate QFT positive (latently infected) contacts and QFT negative (non infected) contacts; and (c) The plasma level of IFN-γ, IL-4, IP-10, MCP-3 and MIP-1β significantly changed after completion of anti-TB treatment.

In the present study we found that the median plasma level of IFN-γ, IL-4, EGF, fractalkine, IP-10 and MCP-3 statistically discriminates ($p<0.005$) patients with active TB and healthy household contacts suggesting that with assay optimization these cytokines and chemokines alone or in combination might serve as markers which can help in diagnosing active TB disease directly from plasma without any additional laboratory manipulation. IFN-γ producing Th1 cells are essential to control mycobacterial replication through activation of macrophages and the prototype Th2 cytokine, IL-4, on the other hand downregulates activation of macrophages. In TB patients the Th1/Th2 bias shifts toward a more marked Th2 response and a lower IFN γ/IL-4 points towards increase disease severity in TB patients (Demissie et al.; 2004). IL-4δ2 is a recently described splice variant of IL-4, which inhibits IL-4 activity and different studies showed that latently infected individuals express high levels of

Th1 cytokines and the IL-4 antagonist IL-4δ2 and individuals with a high IL-4δ2/IL-4 controlled M. tuberculosis infection (Wassie et al.; 2008). EGF is an angiogenic factor and is involved in tissue repair and the higher level of this angiogenic protein in the plasma of TB patients might reflect the disease activity. The higher plasma level of EGF, VEGF and IP-10 is supported by other reports (Juffermans et al.; 1999; Azzurri et al.; 2005; Siawaya et al.; 2009b) who have shown high levels of IP-10, EGF and VEGF in serum or plasma or pleural effusion of TB patients compared with contacts and controls although a recent paper was unable to reproduce the findings (Alessandri et al.; 2006) and showed that pulmonary TB patients and controls had no difference in the plasma concentration of MCP- 1 and IP-10.

Studies have also shown that M. tuberculosis specific stimulation induced IP-10 (CXCL-10) and the sensitivity of detecting M. tuberculosis infection appeared to be better than the Quantiferon test (Ruhwald et al.; 2008; Kabeera et al.; 2010; Belard et al.; 2011) and TB patients have high level of MCP-1, MCP-3, IL-1Rα, and IP-10 responses to TB antigen compared with controls (Ruhwald M et al.; 2009). However, another study did not reproduce this finding and showed that active TB cases produce a higher level of IFN-γ but not IP-10, MCP-2 and IL-2 after stimulation with RD1 selected peptides (Goletti et al.). The IP-10 and MCP-2 response after stimulation with RD1 in TB cases and their contacts was not significantly different but both groups induce higher IP-10 and MCP-2 compared to community controls; on the other hand all the three groups induce similar IL-2 response (Goletti et al.). Another study had also shown that detection of combinations of three host markers or single markers (selected from EGF, sCD40L, MIP-1•, VEGF, TGF-• or IL-1•) in stimulated or unstimulated plasma samples had promising discriminating ability between

latent and active TB. The highest single marker based test sensitivities and specificities were observed for EGF_{Nil} (90 and 84%) and for EGF_{Ag-Nil} (92 and 82%) (Chegou et al.; 2009).

When we grouped the household contacts into latently infected and uninfected based on the QFT-GIT test, the plasma level of EGF, fractalkine, IFN-γ, IL-4, MCP-3 and IP-10 were statistically higher in TB patients than QFT positive or QFT negative subjects, however, none of the cytokines were able to differentiate QFT positive and QFT negative groups. In contrary to this, a recent study conducted in children showed a significantly higher plasma level but not MTB antigen stimulated level of IP-10 in latently infected children than TB patients and community controls (Whittaker et al.; 2008). On the other hand, a recent meta analysis claimed that IP-10 is less affected by infections other than TB and it could be used as a novel diagnostic marker for infection with M. tuberculosis (Belard et al.; 2011). However, the problem in this recent meta analysis is they only compared TB patients, healthy controls and non TB patients and they did not compare the IP-10 level in the latently infected and non infected groups.

We also assessed the effect of HIV on plasma levels of the different cytokines and chemokines of TB cases and we found that the median plasma level of any of the cytokines/chemokines was not affected and there was not statistically different between the two groups in any of the cytokines and chemokines. In our study the plasma level of EGF, fractalkine, IFN-γ, IL-4, MCP-3 and IP-10 were equally high in both groups and can discriminate the TB patients from healthy household contacts irrespective of TB patients' HIV serological status. The detection rate of the old smear microscopy or the newly developed IGRAs is very low in patients with severe TB and immunocompromised patients, which urges the need for exploring new biomarkers for the diagnosis of M.tuberculosis

infection or TB disease particularly in most endemic countries where 50% of TB patients are HIV coinfected.

The median plasma level of IFN-γ, IL-4, MCP-3, MIP-1β and IP-10 changed after treatment. The median plasma level of IL-4 and IP-10 was significantly decreased whereas the level of IFN-γ, MCP-3 and MIP-1β significantly increased after treatment. We know that IFN-γ is a proinflammatory and IL-4 is an anti-inflammatory cytokine and it is reasonable to suggest the increased concentration of IFN-γ and the reduced concentration of IL-4 might be due to the clearance of the actively multiplying bacteria and resolution of the disease by the antituberculosis treatment. Another study also indicated a similar finding where the plasma level of IP-10 decreased and MCP-1 and MIP-1β increased after successful treatment (Azzurri et al.; 2005; Siawaya et al.; 2009a). MCP-1 and MIP-1β are important chemokines and the increased concentration of these chemokines might help in the control of infection by attracting cells to the granuloma. Therefore, the utility of the aforementioned cytokines as indicators of antituberculosis treatment response should now be evaluated in large studies and in different settings and should include the evaluation of patients with treatment failure.

In conclusion, the present study demonstrated that unstimulated plasma EGF, fractalkine, IFN-γ, IL-4, MCP-3 and IP-10 level may have potential in new tools to diagnose both HIV positive and negative TB diseased subjects although none of these markers were able to differentiate latently infected and non-infected individuals. Moreover, we found that the plasma level of IFN-γ, IL-4, MCP-3, MIP-1β and IP-10 changed significantly during anti TB therapy. Further studies are needed to define cut off points for a positive test result, and to explore the potential of these cytokines and chemokines in addition or as alternative to the

existing tests for the development of a rapid, sensitive and user friendly test for the diagnosis of active TB disease in all group of subjects and for monitoring effective anti tuberculosis therapy.

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CHAPTER 5

5. Diversity of Mycobacterium tuberculosis isolates from HIV positive and HIV negative new pulmonary tuberculosis cases in Addis Ababa, Ethiopia

Abstract

Objectives: Understanding the genetic diversity of Mycobacterium tuberculosis is needed for a better understanding of epidemiology of TB and could have implications for development of new diagnostics, drugs, and vaccines. The aim of our study was to understand the genotypes of M. tuberculosis circulating in Addis Ababa.

Method: M. tuberculosis isolates were characterized using spoligotyping and were compared with the SpoIDB4 database of the Pasteur Institute of Guadeloupe.

Result: A total of 53 different patterns were identified among 192 isolates examined. One hundred and sixty nine of the isolates were classified into one of the 33 shared SITs, whereas the remaining 23 corresponded to 20 orphan patterns. Fifty four percent of the isolates were ascribed to the T family, a family that has not been well defined to date. Other prominent families were CAS, Haarlem, LAM, Beijing, and Unknown comprising 26%, 12.5%, 2.6%, 0.5%, and 2.1% respectively. Among HIV positive patients, 10 patterns were observed among 25 isolates, with the remaining one exhibiting a unique pattern. The T (38.5%), H (26.9%), and CAS (23.1%) families were the most common among HIV positive individuals. There was no significant difference in the distribution of strains in HIV positive and HIV negative TB patients except the H family.

Conclusion: The diversity of the M. tuberculosis strains found in this study was very high and there was no difference in the distribution of families in HIV positive and HIV negative TB patients. Tuberculosis transmission in Addis Ababa is due to only the modern M. tuberculosis families (CAS, LAM, T, Beijing, Haarlem, and U) despite the historical African origin of M. tuberculosis.

5.1 Introduction

Tuberculosis (TB) continues to be a major public health problem in Ethiopia. Currently Ethiopia is rated 7th among the 22 high TB-burdened nations of the world, with a prevalence of 394 per 100,000 population in the year 2010 (WHO; 2011). This situation has been worsened by the country's HIV/AIDS epidemic; HIV prevalence was 3.5% in 2009 and among TB patients, 15% were co-infected with HIV (1), and TB is the leading cause of mortality among HIV positive patients (FMoH.; 2009). Despite the historical origin of TB in the region and the high TB burden in the country, very limited information is available on the genetic diversity of M. tuberculosis strains and the impact of HIV disease on this diversity in the country.

Molecular typing techniques have been extensively used to speciate strains of M. tuberculosis involved in TB infections, providing insights into dissemination dynamics, evolutionary genetics, and detection of suspected outbreaks and person to person transmission (Brosch et al.; 2002a). Although recent studies are recommending the use of robust markers such as single nucleotide polymorphisms (SNP) or large sequence polymorphisms (LSP) for a better understanding of strain lineages (Comas and Gagneux; 2011), Insertion sequence (IS) 6110 restriction fragment length polymorphism (IS6110 RFLP) has in the past served as a gold standard typing method (van Embden et al.; 1993; Kremer et al.; 1999). However, it is costly, time consuming, and not easily standardized across laboratories.

An alternative technique is a PCR amplification based technique, spacer oligonucleotide typing (spoligotyping), which analyses polymorphisms of direct repeat (DR) regions. The

technique is relatively simple, quick, and reliable. The method has been extensively used for simultaneous detection and typing of M. tuberculosis (Kamerbeek et al.; 1997a). Currently spoligotyping has been successfully applied to understand the emerging problem of multidrug-resistant (MDR) and extensively drug resistant (XDR) TB and to investigate the virulence of different strains of M. tuberculosis, as well as to better understand the epidemiology of TB (Affolabi et al.; 2009; Dong et al.; 2010; Rosales et al.; 2010; Viegas et al.; 2010; Afanas'ev et al.; 2011; Groenheit et al.; 2011). A recent study on molecular analysis of M. tuberculosis in Ethiopia described the diversity of strains although it failed to address the pattern in TB-HIV co-infection (Agonafir et al.; 2010). The present study aimed to provide additional insight into the diversity of clinical isolates in HIV positive and HIV negative patients in Addis Ababa, Ethiopia using spoligotyping and to compare the patterns obtained with those available in the international spoligotyping database, SpolDB4.0 of the Pasteur Institute of Guadeloupe.

5.2 Materials and Methods

M. tuberculosis strains and DNA isolation

A total of 192 M. tuberculosis isolates were collected between July 2009 and June 2010 from smear positive new TB cases at four different health centres in Addis Ababa. Demographic, epidemiologic, and clinical information for all patients were collected using a prestructured questionnaire. All participants were tested for antibodies to HIV-1 and -2 after pre- and post-test counselling using a rapid test (Stat pack, KHP and Unigold as a tie braker) as per the Ethiopian National guide line. All sputum samples from TB cases were cultured for mycobacteria following the protocol described in the methodology section (2.4.6). M. tuberculosis isolates were identified using PCR-based genotyping with previously described

methods for RD9 deletions and genotyping was done using spoligotyping as described in the methodology part of the thesis (2.4.7 and 2.4.8).

The spoligo patterns which were prepared in binary and octal were entered and determined by comparing the spoligotyping results with already existing designations in the international spoligotyping database, SpolDB4.0 (Brudey et al.; 2006) (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo). Families and subfamilies where assigned for patterns which were not registered by using the SpotClust program, which was built on the SpolDB3 database (http://cgi2.cs.rpi.edu/ bennek/SPOTCLUST.html).

5.3 Result

5.3.1 Demographic information

Of the total 192 patients sampled, 26 (13.5%) were HIV positive, 136 (70.8%) were HIV negative and the remaining 30 (15.6%) were not tested for HIV. The mean age was 28.7 years (range 18—72). There were slightly more male participants than female (56.9%). For the HIV-positive patients, the mean CD4 cell count at the time of presentation was 312 ±22.4 cells/•L.

5.3.2 Genetic diversity and family assignment

Among the 192 typed isolates, 169 (88%) were classified into one of the 33 STs according to SpolDB4.0. The remaining 23 isolates generated 20 different spoligotypes with 3 new shared spoligotypes (n=2 in each cluster) that had not been previously described in the database. Among the total 53 spoligotype patterns characterized in the present study, 20 patterns (including the 3 new clustered patterns) corresponding to clusters with 2-41 isolates per cluster were identified, accounting for a very high clustering rate of 88% (169/192). Out of the 33 patterns that did not form clusters, 17 represented true orphan patterns that did not previously exist in SpolDB4.0 and 16 represented pseudo-orphans which were present as singles in this study but found in SpolDB4.0 (The ST and Family distribution of clustered strains is summarized in Figure 5-1).

Webdings Format	Octal Code	ST	Family	No. of strains in this study	% of strains in this study
c c c c c c c c c c c c c c c c c c c c c c c c c c c c c c c c c c g g g g g g g g g	000000000003771	1	BEIJING	1	0.5
g g g c c c c g g g g g g g g g g g g g g c c c c c c c c c c c c c c c g g g g g g g g g	703777700003771	142	CAS	2	1
g g g c c c c g g g g g g g g g g g g g g g c c c c c c c c c c c c c c c c c c c c c c c	703777740000000	1264	CAS	1	0.5
g g g c c c c c g g g g g g g g g g g g g g g c c c c c c c c c c c c c c g g c c g g g g g	703777740003171	25	CAS_DELHI	18	9.4
g g g c c c c g g g g g g g g g g g g g g g c c c c c c c c c c c c c c c g g g g g g g g g	703777740003771	26	CAS_DELHI	5	2.6
g g g c c c c g c g g g g g g g g g g g g g c c c c c c c c c c c c c g g c c g g g g g	702777740003171	140	CAS_DELHI	1	0.5
g g g c c c c g g g g g g g g g g g g g g g c c c c c c c c c c c c c g g g c c g g g g	703777740003471	247	CAS_DELHI	1	0.5
g g c c c c g g c g g g g g g g g g g c c c c c c c c c c c c c c c g g g g g g g g g	606777000003771	21	CAS_KILI	14	7.3
g g g g g g g g g g g g g g g g g g g g g g g g g c c c c c g c c c c g g g g g g g	777777774020771	47	H1	1	0.5
c c c c c c c c c c c c c c c c c c c c c c c c c g g g g g g c g c c c c g g g g g g g	000000007720771	3	H3	1	0.5
g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g c g c c c c g g g g g g g	777777777720771	50	H3	7	3.6
g g g g g g g g g g g g g g g g g g g g g g g g g g g c g g g c g c c c c g g g g g g g	777777775720771	121	H3	4	2.1
g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g c g c c c c g g c c g g g	777777777720631	134	H3	2	1
g g c g g g g g g g g g g g g g g g g g g g g g g g g g g g g c g c c c c g c g g g g	677777777720571	699	H3	1	0.5
g g g g g g g c c g g g g g g g g g g g g g g g g g g c c c g c c c c g g g g g g g	774777777420771	262	H4	1	0.5
g g g g g g g g g g g g g g g g g g g g g g g g g g g c c c g c c c c g g g g g g g	777777777420771	777	H4	6	3.1
g g g g g g g g g g g g g g g g g g g g g g g g g g g c c c g c c c c g g g c g g g	777777777420731	817	H4	1	0.5
g g g g g g g g g g g g g g g g g g g c c c c c g c c g g g g g c c c c g g g g g g g	777777404760771	41	LAM7_TUR	1	0.5
g g g g g g g g g g g g g g g g g g g c c c c g g g g g g g g c c c c g g g g g g g	777777607760771	42	LAM9	2	1
g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g c c c g g g g g g g g	777777777760771	53	T1	21	10.9
g g g g g g g g g g g g g g g g g g g g g g g c g g g g g g g g c c c c g g g g g g g	777777767760771	373	T1	1	0.5
g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g c c c c g g g c g g	777777777760751	612	T1	4	2.1
g g g g g g g g g g g g g g g g g g g c c c c c g g g g g g g c c c c g g g g g g g	777777403760771	1688	T1	1	0.5
g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g c c c c g g g c g g g	777777777760731	52	T2	3	1.6
g g g g g g g g g g g g g g g g c g g g g g g g g g g g g g g c c c c g g g c g g g	777775777760731	584	T2	4	2.1
g g g g g g g g g g g g g g g g g g c g g g g g g g g g g g g g c c c c g g g c g g g	777777377760731	1077	T2	1	0.5
g g g g g g g g g g g c g g g g g g g g g g g g g g g g g g g c c c c g g g c g g g	777737777760731	73	T2-T3	1	0.5
g g g g g g g g g g g c g g g g g g g g g g g g g g g g g g g c c c c g g g g g g g	777737777760771	37	T3	14	7.3
g g g g g g g g c c c c c c c c c c g g g g g g g g g g g g g c c c c g g g g g g g	777000377760771	149	T3_ETH	41	21.4
g g g g g g g g c c c c c c c c c c g g c g g g g g g g g g g c c c c g g g c g g g	777000377760731	345	T3_ETH	3	1.6
g g g g g g g g g g g g g g g g g g g g g g g c g g g g g g g g g c c c c g g g g g g g	777777757760771	44	T5	1	0.5
g g g c c c c c c c c c c c c c c c c c c c c c g g g c c g g g g g g g g g g g g g	700000007177771	910	U	3	1.6
g g g g g g g g g g g g g g g g g g g g c c c c g g g g g c c c c c c c c c c c g g g	777777607600031	1689	U	1	0.5
g g g g g g g g g c c c c c c c c c c g g c g g g g g g g g g c c c c g g g g g g g	777000337760771	Orphan	Orphan	1	0.5
g g g g g g g g g g g g g g g g g g g g c c c c c c c g c c g g g g g c c c c g g g g g g	777777004760771	Orphan	Orphan	1	0.5
g c c g g g g g g c g g g c g g g g c g c g g g g g g g c c c c g c g g g g g g g g	477357277413771	Orphan	Orphan	1	0.5
g g g c c c c g g g g c g g g g g g g g g c c c c c c c c c c c c c g g c c g g g g g	703677740003171	Orphan	Orphan	2	1
g g g c c c c g g c g g g g g g g g g c c c c c c c c c c c c c c c g g g g g g g g g	703377400003771	Orphan	Orphan	2	1
g g g c c c c g g c g g g c c g g g g g g g c c c c c c c c c c c c c g g g g c g g g	703347740003671	Orphan	Orphan	1	0.5
g c g c c g g c g c c c c c c c c c c g c c g g g g c g g g g c c c c c c g c g g c g	515000236740261	Orphan	Orphan	1	0.5
g g g g g g g g g g g g g g g g g g g g c c c c g g g g g g g g g c c c c g g g c c c g	777777607760701	Orphan	Orphan	1	0.5
g g g g c g g g g g g g g g g g g g c g g g g g g g g g g g g g c c c c g g g g g g g	757777377760771	Orphan	Orphan	1	0.5
g g g g g g g g g c g g g g g g g g g g g g g g g g g g g g g c c c c g g g g c g g	777377777760751	Orphan	Orphan	1	0.5
g g g c c c c c c c c c c c c c c c c c c c c c g c c c c g g c c g g g g g g g c c c	700000004147740	Orphan	Orphan	2	1
g g g g g g g g g g g c c c g g g g g g g c g g g c c c c g c c c c g g g g g g g	777707767020771	Orphan	Orphan	1	0.5
g g c g g g g g g g g g g g g g g g g g g g g g g g g g g c g g c c g c c c c g c g g g g g	677777776620571	Orphan	Orphan	1	0.5
g g g c c c c g g g g g g g g g g g g g g g g c c c c c c c c c c c c c g g g g g g g g	703777760003771	Orphan	Orphan	1	0.5
c c c c c c g g c g g g c g g g g g g g g g g g g g g g g g g g c c c c g g g g g g g	006737777760771	Orphan	Orphan	1	0.5
g g g g g g g g g g g g g g c c c c c g g g g g c c c c c c c c c c c c c c c c c c c	777760370000000	Orphan	Orphan	1	0.5
g g g g g c c g g g g g g g g g g g g g g g g g g g g g g c c c g c c c c g g g g g g g	763777777420771	Orphan	Orphan	1	0.5
g g g c c c c g g c g g g g g g g g g c c c c c c c c c c c c c c c c g g g g g g g g	703377600001771	Orphan	Orphan	1	0.5
g g g c 1 c c g g g g g c g g g g g g g g g c c c c c c c c c c c c c g c c g g g g g	703737740001171	Orphan	Orphan	1	0.5
g g g g g g g g g g g g g c g g g g g g g g g c g g g g g g g c g c c c c g g g g g g g	777757767720771	Orphan	Orphan	1	0.5

Figure 5-1 Spoligotype pattern of clustered M. tuberculosis strains.
The black and white boxes indicate the presence and absence, respectively, of the specific spacer at position 1 to 43 in the DR locus. ST= Shared Type; CAS = Central Asian; LAM = Latin American-Mediterranean; H=Haarlem, U= Unknown

Family assignment revealed that the T family represented the most predominant family in our study, constituted of 104, (54.2%) isolates with the following distribution: T3_ETH (44, 22.9%); T1 (34, 17.7%), T2 (8, 4.2%); T2-T3 (1, 0.5%), T3 (16, 8.3%) and T5 (1, 0.5%). The CAS Families (50, 26%) were the second most dominant family with CAS1_DELHI (25, 13%); CAS_KILLI (14, 7.3%) and CAS (9, 4.7%). Other families found were Haarlem (H) (24, 12.5%)

with the following distribution: H1 (1, 0.5%), H3 (15, 7.8%), H4 (8, 4.2%); the LAM Family (5, 2.6%): LAM9 (4, 2.1%), LAM7_TUR (1, 0.5%); the Beijing Family (1, 0.5%); Family 34 (1, 0.5%); Family 36 (1, 0.5%) and 4 (2.1%) strains were Undetermined (the main results of the spoligotyping analysis are summarized in Table 5-1).

Table 5-1 Family and family distribution and associated ST for each isolate in the study

Family	Family	Shared Type, Number of isolates
Beijing n=1 (0.5%)	Beijing n=1	1, n=1
CAS n=50 (26%)	CAS n=11	142, n=2
		1264, n=1
		Orphan, n=8
	CAS1_KILI n=14	21, n=14
	CAS_DELHI n=25	25, n=18
		26, n=5
		140, n=1
		247, n=1
H n=24 (13.2%)	H1 n=1	47, n=1
	H3 n=15	3, n=1
		50, n=7
		121, n=4
		134, n=2
		699, n=1
	H4 n=8	262, n=6
		777, n=1
		817, n=1
LAM n=5 (2.6%)	LAM7_TUR n=1	41, n=1
	LAM9 n=4	42, n=2
		Orphan, n=2
T n=104 (54.2%)	T1 n=34	53, n=21
		373, n=1
		612, n=4
		1688, n=1
		Orphan, n=7
	T2 n=8	52, n=3
		584, n=4
		1077, n=1
	T2-T3 n=1	73, n=1
	T3 n=16	37, n=14

		Orphan, n=2
	T3_ETH n=44	149, n=41
		345, n=3
	T5 n=1	44, n=1
EAI n=1 (.5%)	EAI5	Orphan, n=1
Family 34 n=1 (0.5%)		Orphan, n=1
Family 36 n=2 (1%)		Orphan, n=2
U n=4 (2.1%)	U n=4	910, n=3
		1689, n=1

Among the total 33 Shared types which comprised 169 isolates, a total of 126 isolates (65.6%) from 8 clusters which contained 5-41 isolates per cluster formed the major isolates and 43 isolates (22.4%) from 25 clusters which contained 1-4 isolates per cluster formed the minor isolates. Sixteen isolates were pseudo orphans whereas 23 isolates generated 20 unique spoligotypes that had not been previously described in the database. The spoligotype pattern of the orphan isolates is summarized in figure 5-2.

Webdings Format	Octal Code	ST	Family*	Probability**	No. of strains in this study	% of strains in this study
ggggggggccccccccccgcgggggggggcccgggggg	777000337760771	Orphan	T3	0.99	1	0.5
gggggggggggggggggcccccgcgggggcccgggggg	777777004760771	Orphan	LAM9	0.98	1	0.5
gcgggggggcgggcgggcgggggggcccgggggggg	477357277413771	Orphan	EAI5	0.99	1	0.5
gggcccgggcgggggggggcccccccccccccgggggg	703677740003171	Orphan	CAS	0.99	2	1
gggccccgggggggggcccccccccccccgggggggg	703377400003771	Orphan	CAS	0.99	2	1
gggccccggcgggccgggggcccccccccccgggggg	703347740003671	Orphan	CAS	0.99	1	0.5
gcgccggcgccccccccccgccgggcgggcccccgcggcg	515000236740261	Orphan	T3	0.99	1	0.5
gggggggggggggggggcccgggggggcccccgggccg	777777607760701	Orphan	LAM9	0.99	1	0.5
ggggcgggggggggcgggggggggcccccgggggg	757777377760771	Orphan	T1	0.99	1	0.5
gggggggggcgggggggggggggggggcccccgggcgg	777377777760751	Orphan	T1	0.99	1	0.5
gggcccccccccccccccccccccgcccccggccgggggggccc	700000004147740	Orphan	FAMILY 36	0.99	2	1
gggggggggggccccgggggggcgggccccgcccccgggggg	777707767020771	Orphan	T1/H3	0.22/0.77	1	0.5
ggcgggggggggggggggggggggcggccgccccgggggg	677777776620571	Orphan	T1/H3	0.22/0.77	1	0.5
gggcccccgggggggggggggcccccccccccgggggggg	703777760003771	Orphan	CAS	0.95	1	0.5
ccccccggcgggcgggggggggggggggcccccgggggg	006737777760771	Orphan	T1	0.99	1	0.5
gggggggggggggcccccgggggcccccccccccccccccc	777760370000000	Orphan	FAMILY 34	0.99	1	0.5
gggggccgggggggggggggggggggggcccgccccgggggg	763777777420771	Orphan	T1/H3	0.22/0.99	1	0.5
gggccccggcgggggggggcccccccccccccgggggggg	703377600001771	Orphan	CAS	0.99	1	0.5
gggcccccgggggcgggggggggcccccccccccccgccgggggg	703737740001171	Orphan	CAS	0.99	1	0.5
ggggggggggggcggggggggcgggggcgcccccgggggg	777757767720771	Orphan	T1/H3	0.22/0.77	1	0.5

Figure 5-2 Spoligotype pattern of orphan M. tuberculosis strains from HIV positive and HIV negative patients.
The black and white boxes indicate the presence and absence, respectively, of the specific spacer at position 1 to 43 in the DR locus * SpotClust program-assigned family. **Probability that the spoligotype pattern belongs to the family

5.3.3 Spoligopattern of isolates from HIV positive TB cases

For the HIV positive patients, spoligotyping produced a total of 11 different patterns. Among the 26 patients, 25 (92.3%) were classified into one of the 10 shared international types (SITs) according to SpolDB4.0. The remaining 1 isolate generated a unique spoligotype pattern that had not been previously described in the database. Among the total 11 spoligotype patterns characterized in the present study, 5 patterns corresponding to clusters with 2-5 isolates per cluster were identified and the remaining 5 patterns were pseudo orphans. One spoligotype represented a true orphan pattern that did not previously exist in SpolDB4.0. Family assignment in HIV positive subjects also revealed that the ill defined T family constitutes the most predominant family (38.5%) followed by H Family constituting 26.9% and CAS_DELHI (23.1%). Although the small sample size of HIV positive subjects did not allow us to make a strong comparison with HIV negative subjects, we did not find a significant difference in the distribution of the different families in the two groups. The spoligo pattern of the isolates from HIV positive subjects is summarized in figure 5-3.

Webdings Format	Octal Code	ST	Family	No. of strains in this study	% of strains in this study
g g g c c c c g g g g g g g g g g g g g c c c c c c c c c c c c g g c c g g g g g	703777740003171	25	CAS_DELHI	4	15.4
g g g c c c c c g g g g g g g g g g g g g c c c c c c c c c c c c c g g g g g g g g g	703777740003771	26	CAS_DELHI	2	7.7
g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g c g c c c c c g g g g g g g	777777777720771	50	H3	3	11.5
g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g c c c g c c c c c g g g g g g g	777777777420771	777	H4	3	11.5
g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g c c c g c c c c c g g g c g g g	777777777420731	817	H4	1	3.8
g g g g g g g g g g g g g g g g g g g g g c c c c c g c c g g g g g c c c c c g g g g g g g	777777404760771	41	LAM7_TUR	1	3.8
g g g g g g g g g g g c g g g g g g g g g g g g g g g g g g g g g c c c c g g g c g g g	777737777760731	73	T2-T3	1	3.8
g g g g g g g g g g c g g g g g g g g g g g g g g g g g g g g g g c c c c g g g g g g g	777737777760771	37	T3	4	15.4
g g g g g g g g g c c c c c c c c c c g g g g g g g g g g g g g g c c c c g g g g g g g	777000377760771	149	T3_ETH	5	19.2
g g g c c c c c c c c c c c c c c c c c c c c c g g g c c g g g g g g g g g g g g g g	700000007177771	910	U	1	3.8
g g g g g g g g g g g g g g g g g g g g g c c c c g g g g g g g g c c c c g g g c c c g	777777607760701	Orphan	Orphan	1	3.8

Figure 5-3 Spoligotype pattern of M. tuberculosis strains from HIV positive subjects.
The black and white boxes indicate the presence and absence, respectively, of the specific spacer at position 1 to 43 in the DR locus.

5.4 Discussion

Analysis and timely updates of the M. tuberculosis strain distributions in a given country and comparisons to worldwide patterns provides insights into transmission mechanisms, emergence of drug resistance and particularly virulent strains, and potentially information relevant to the development of new diagnostics, drugs and vaccines.

This study has been undertaken in HIV positive and HIV negative patients in order to gain a better understanding into the population structure of M. tuberculosis in Addis Ababa. Spoligotyping was used as a primary typing tool because of its ease of use, straightforward coding and existence of an international database of global isolates for comparative analysis.

There are variations in different areas with regard to the distribution of the dominant families (Brudey et al.; 2006). In our study, the majority of the isolates (83.2%) belonged to three major families: T family (54.2%), CAS (26%) and H (13.2%) and there was no difference in the distribution of families in HIV positive and HIV negative subjects (Figure 5-4).

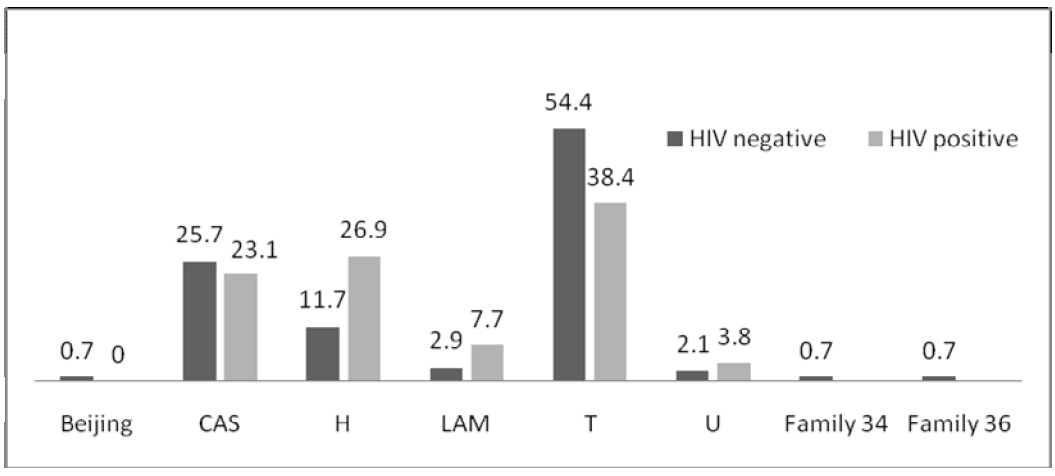

Figure 5-4 M. tuberculosis spoligotype families in HIV positive and HIV negative patients

Although the T family was the most prevalent in this study, it is believed that the T family does not represent a particular family and that it encompasses families which are not grouped into one of the other families (Brudey et al.; 2006). Hence it is frequently referred to as the "ill-defined" family of M. tuberculosis that is found worldwide.

The T3_ETH was the dominant family and previous reports also showed this spoligotype present in high proportion in Ethiopia (Agonafir et al.; 2010) and it is believed to be specific for Ethiopia and this ST has not been reported from any other country (Brudey et al.; 2006). The CAS family, which was the second most frequent spoligotype in this study, had been reported in previous studies in Ethiopia (Agonafir et al.; 2010) and also prevalent in Tanzania (Kibiki et al.; 2007) with CAS1_KILI predominant and in Kenya 35.6% of 73 isolates were of the CAS family (Githui et al.; 2004). The H family constituted 13.2% of all families in our study and it is wide-spread in different geographical regions of the world such as Asia, Europe, Middle East and Africa has been documented with some reports associated with outbreaks in Argentina (Ritacco et al.; 1997) and in Czech Republic (Kubin et al.; 1999).

The other families were LAM and Beijing comprising 5 and 1 isolates respectively. Among the LAM family we had two strains, LAM7_TUR and LAM9. The LAM7_TUR is believed to be a strain restricted to Turkey (Zozio et al.; 2005) where as LAM9 has been reported from different parts of the world including Africa. SIT1 corresponds to the Beijing genotype is the first report from Ethiopia but it needs to be confirmed with other molecular methods as spoligotyping has limitations for genotypic classification. The Beijing family is a common strain in East Asia but recent reports indicated this strain is distributed all over the world and it is taken as an indicator of recent introduction into a particular area. In recent years

several countries reported an increase over time of the proportion of TB due to Beijing genotype strains including countries in the region (Githui et al.; 2004; Kibiki et al.; 2007).

Generally, despite the presence of predominant shared types, the diversity of the M. tuberculosis found in the present study is very high; 192 isolates produced 53 different spoligotypes. The families observed in this study represented more than 85.5% of the total 62 families currently registered in the fourth international spoligotyping database, SpoID4. Spoligotyping has less discriminatory power; therefore, for a better understanding and confirmation of the diversity, more discriminative techniques will be required. Moreover, the effect of this diversity on M. tuberculosis transmission and whether strain differences might elicit different immune responses is not clear and it needs further study. Different studies have indicated that M. tuberculosis strains differ in their immunogenicity and virulence (de Jong et al.; 2008; Nicol and Wilkinson; 2008; Portevin et al.; 2011). It is also advisable to do follow up studies in order to obtain a clear molecular-epidemiological overview of the Beijing and other strains. This study was done in a few health centers in the capital city of Addis Ababa, so our findings here may not be representative of the entire country; therefore, we recommend future wider studies with a better molecular method with a better discriminative power like SNP typing to elucidate strain diversity in detail within Ethiopia.

5.5 References

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CHAPTER 6

6. Plasma levels of IL 4 differ in patients infected with different modern lineages of M. tuberculosis

Abstract

Background: The genetic diversity of Mycobacterium tuberculosis may have important clinical consequences. Epidemiological evidence from tuberculosis outbreaks suggested that the virulence, site of disease and capacity for transmission is not the same in all genotypes of M. tuberculosis. We analysed the plasma cytokine levels of untreated, first-episode pulmonary tuberculosis patients infected with different strains of M. tuberculosis to test the hypothesis that immune responses would be linked to the bacterial genotype.

Methods: Spoligotyping was carried out for genotyping and we used Luminex technology to measure 17 cytokines (EGF, fractalkine, GM-CSF, IFN-γ, IL-1, IL-10, IL-12, IL-17, IL-4, IL-7, IL-9, IP-10, MCP-1, MCP-3, MIP-1β, TNF and VEGF) from plasma samples of tuberculosis patients.

Result: The level of IL-12 (p40), IL-4, IL-7 and MIP-1betta were higher in patients infected with lineage 3 than lineage 4, however, it was only IL-4 which showed statistically significant difference ($p<0.05$) between the two lineages. We further grouped the lineages into different M. tuberculosis families (CAS, H and T family) and we found that the plasma level of IL-4 was significantly higher in patients infected with the CAS family ($p<0.05$) in comparison with T and H families. However there was no any difference between T and H family.

Conclusion: There is a significant difference in the plasma levels of IL-4 between patients infected with different lineages and families of the modern lineage. Whereas it is unknown if such differential cytokine responses are responsible for different clinical outcomes in hosts infected with these strains, further investigation should address this issue to improve our understanding of mechanisms for pathogenicity..

6.1 Introduction

Tuberculosis remains a significant public health problem infecting one third of the world's population resulting 8.8 million cases and 1.45 million deaths annually (WHO; 2011). The factors that lead to the considerable variability in the outcome of M. tuberculosis infection are complex and incompletely understood. Host genetics and environmental factors like prior exposure to non-pathogenic mycobacteria, HIV infection, advanced age, malnutrition, alcohol abuse, diabetes and corticosteroids have been associated with tuberculosis disease (Bellamy; 2003). There are studies that indicate that the different clinical outcomes of M. tuberculosis infection could be associated with the genotype of M. tuberculosis. There is growing evidence that the genetic diversity of M. tuberculosis may have important clinical consequences (Nicol and Wilkinson; 2008; Coscolla et al.; 2010).

Epidemiological evidence from tuberculosis outbreaks suggested that the virulence, site of disease and capability of transmission is not the same in all genotypes of M. tuberculosis (Hirsh et al.; 2004; Gagneux et al.; 2006). A number of studies have described the ''Beijing family'' to be hyper virulent with a reduced immune response leading to higher bacillary load and enhanced dissemination with rapid progression to severe disease in humans and experimental animals (Lopez et al.; 2003; Manca et al.; 2004; Hanekom et al.; 2007; Tanveer et al.; 2009).

A study in Gambia showed that progression to clinical disease was significantly less in individuals exposed to the ancient lineage compared to the modern lineage (de Jong et al.; 2008) and another study in Madagascar reported significantly lower interferon-γ production by peripheral blood T cells in individuals infected with the modern lineage (Rakotosamimanana et al.; 2010). Moreover, a recent study by Portevin et al also showed that measurement of cytokines from culture supernatants harvested 24 hours after

infection of human peripheral blood monocyte derived macrophages revealed clear differences in the level of proinflammatory cytokines produced by a single donor in response to different strains (Portevin et al.; 2011).

In the present study, we used plasma samples from new pulmonary tuberculosis patients to test the hypothesis that immune response would be linked to the infecting genotype by measuring the plasma levels of different cytokines and chemokines.

6.2 Materials and Methods

Patient recruitment

A total of 75 subjects with microbiologically confirmed new smear positive pulmonary TB patients attending Arada, T/Haimanot, Kirkos and W-23 health centres, Addis Ababa, who were infected with one of the three major families, T, H and CAS (previous chapter) were randomly selected from the total of 192 patients.

Multiplex cytokine analysis, mycobacterium culture and DNA extraction, deletion typing, spoligotyping and spoligo database comparison was done as indicated in the methodology section of the thesis (2.4.3, 2.4.6-2.4.9).

Statistical analysis

The data were analyzed using Graph Pad Prism software, version 4.0. Nonparametric Mann–Whitney U tests were performed to find the significance of the observed differences in each parameter in TB and other groups. A P value less than 0.05 was considered statistically significant.

6.3 Result

6.3.1 Genetic diversity and family assignment

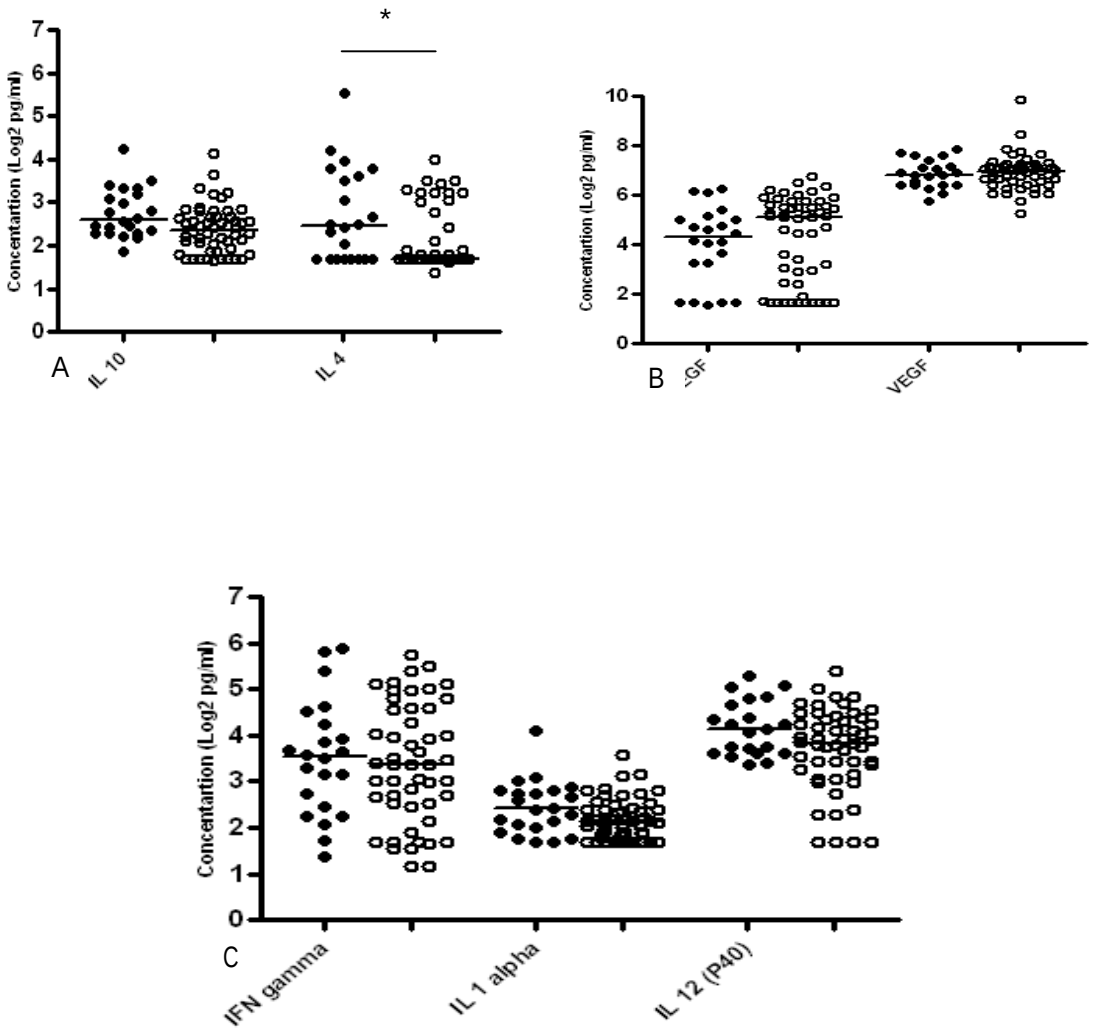
A total of 75 TB patients infected with M. tuberculosis belonging to lineages 3 and lineage 4 were selected randomly from 192 patients. The lineage 3 comprises 22 CAS strains and lineage 4 comprises 35 T and 18 H strains. Of all the total 75 patients, 26 (34.6%) were females. The mean age was 28.7 years (range 18—64) and 31.7 years (range 18-59) for females. The representative spoligopattern is indicated in figure 6-1.

Webdings Format	Octal Code	Family	Lineage
g g g c c c c g g g g g g g g g g g g c c c c c c c c c c c c g g g g g g g g g	703777700003771	CAS	3
g g g c c c c g g g g g g g g g g g g c c c c c c c c c c c c c c c c c c c c c	703777740000000	CAS	3
g g g c c c c g g g g g g g g g g g g c c c c c c c c c c c c g g c c g g g g g	703777740003171	CAS	3
g g g c c c c g g g g g g g g g g g g c c c c c c c c c c c c g g g g g g g g g	703777740003771	CAS	3
g g c c c c g g c g g g g g g g g g c c c c c c c c c c c c c g g g g g g g g g	606777000003771	CAS	3
g g g g g g g g g g g g g g g g g g g g g g g g c c c c c c g c c c c g g g g g g g	777777774020771	H	4
c c c c c c c c c c c c c c c c c c c c c c c c g g g g g g c g c c c c g g g g g g g	000000007720771	H	4
g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g c g c c c c g g g g g g g	777777777720771	H	4
g g g g g g g g g g g g g g g g g g g g g g g g g g g g g c c c g c c c c g g g g g g g	777777777420771	H	4
g g g g g g g g g g g g g g g g g g g g g g g g g g g g g c c c g c c c c g g g c g g g	777777777420731	H	4
g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g g c c c c g g g g g g g	777777777760771	T	4
g g g g g g g g g g g g g g g c g g g g g g g g g g g g g g g c c c c g g g c g g g	777775777760731	T	4
g g g g g g g g g g g g g g g g c g g g g g g g g g g g g g g c c c c g g g c g g g	777777377760731	T	4
g g g g g g g g g g g g c g g g g g g g g g g g g g g g g g g c c c c g g g g g g g	777737777760771	T	4
g g g g g g g g g c c c c c c c c c c g g g g g g g g g g g g g g g c c c c g g g g g g g	777000377760771	T	4
g g g g g g g g g c c c c c c c c c c g g g g g g g g g g g g g g g c c c c g g g c g g g	777000377760731	T	4

Figure 6-1 Representative spoligotype pattern of M. tuberculosis strains.
The black and white boxes indicate the presence and absence, respectively, of the specific spacer at position 1 to 43 in the DR locus. CAS = Central Asian; T = ill defined family; H=Haarlem.

6.3.2 Plasma cytokine and chemokine levels of patients infected with different M. tuberculosis lineages

Plasma samples from 75 TB patients infected with strains belonging to lineage 3 (CAS) (n=22) and lineage 4 (H and T) (n=53) were analysed and we compared the plasma cytokine and chemokine levels between the two lineages. The level of IL-12 (p40), IL-4, EGF, IP-10 and MIP-1β were higher in patients infected with lineage 3, however, it was only IL-4 which was significantly different between the two lineages (P=0.01250.05) (figure 6-2).



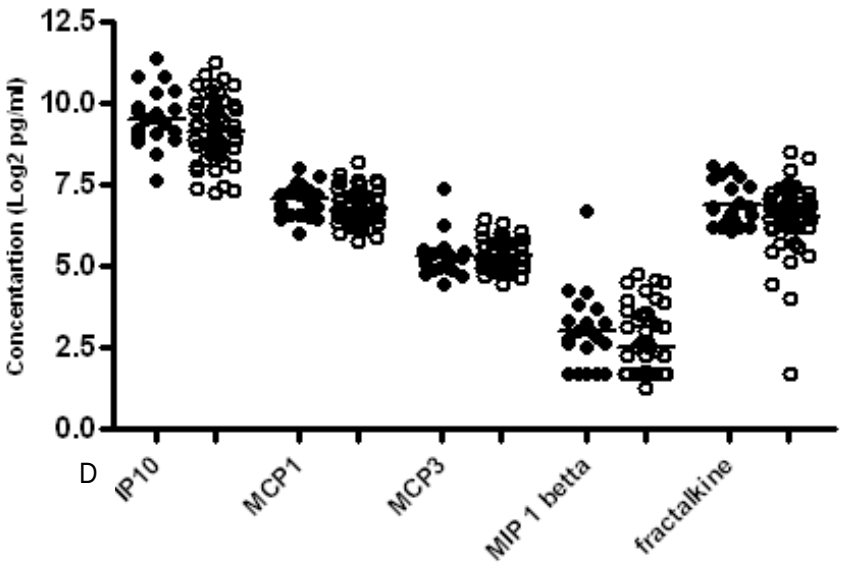
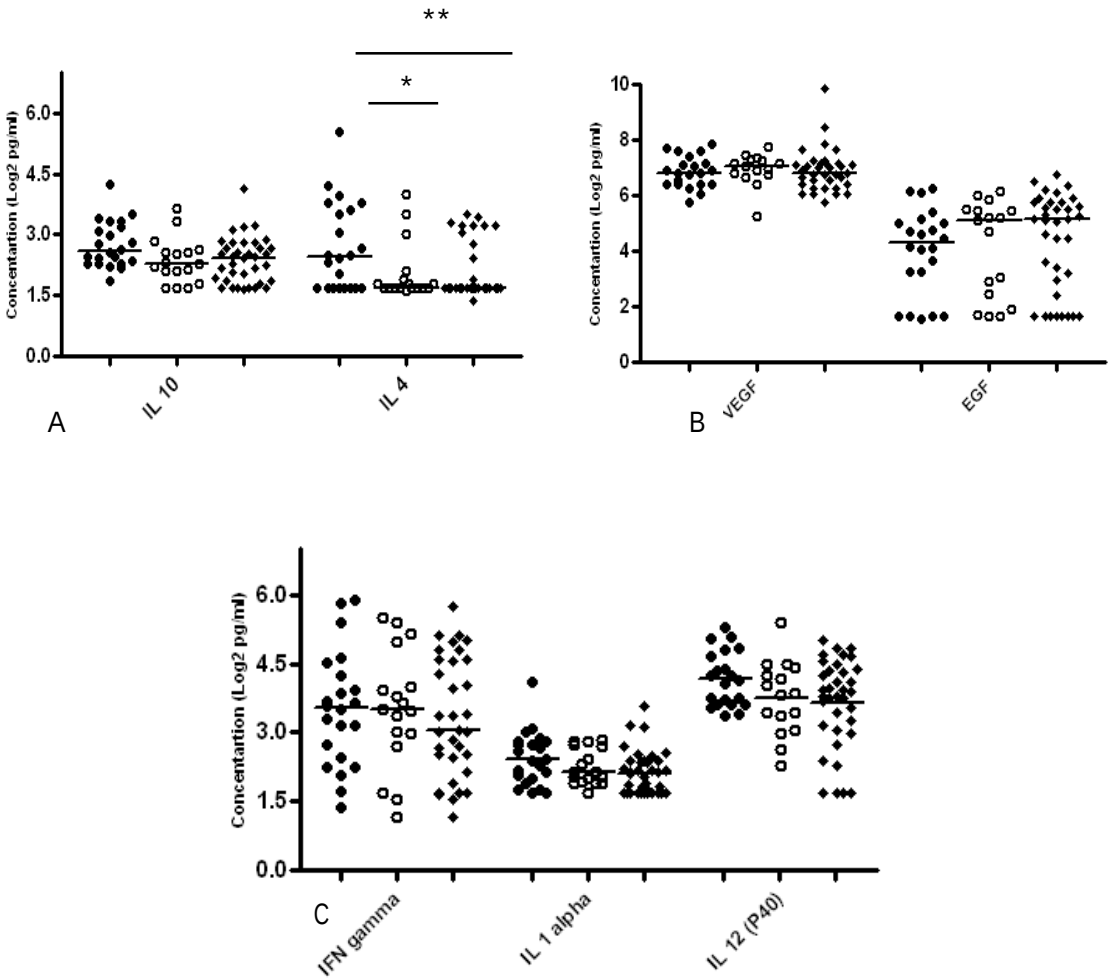


Figure 6-2 Plasma cytokine and chemokine levels in TB cases infected with different lineages.

Plasma samples from TB cases infected with lineage 3 (n=22), and lineage 4 (n=43) were assessed by multiplex cytokine analysis. A) Anti-inflammatory cytokines (IL-10 and IL-4), B) Growth factors (EGF and VEGF), C) Pro-inflammatory cytokines (IFN-γ, IL 12(p40), and TNF) and D) Chemokines (IP-10, MCP-1, MCP-3, MIP-1β and fractalkine). Horizontal lines indicate median levels of TB cases infected with lineage 3 (filled circles), and lineage 4 (open circles). Data were analysed using the nonparametric Mann-Whitney test with p-values indicating significant differences after transformation of data to Log2 values.*P<0.05.

6.3.3 Plasma cytokine and chemokine levels of patients infected with different M. tuberculosis families

We further grouped the lineages into different M. tuberculosis families: 22 CAS, 35 T and 18 H family and we found that only the level of IL 4 was significantly higher in plasma samples infected with the CAS family in comparison with H (P=0.024) and T (P<0.001)family. However, there was no difference between T and H family (Figure 6-3).



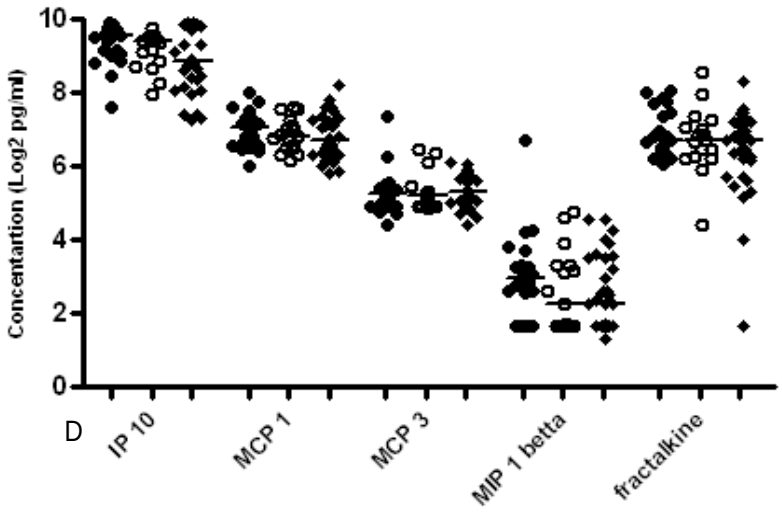


Figure 6-3 Plasma cytokine and chemokine levels in TB cases infected with different strains.
Plasma samples from TB cases infected with CAS (n=22), H (n=18) and T families (n=35) were assessed by multiplex cytokine analysis. A) Anti-inflammatory cytokines (IL-10 and IL-4), B) Growth factors (EGF and VEGF), C) Pro-inflammatory cytokines (IFN-γ, IL 12(p40), and TNF) and D) Chemokines (IP-10, MCP-1, MCP-3, MIP-1β and fractalkine). Horizontal lines indicate median levels of TB cases infected with the CAS family (filled circles), H family (open circles) and T families (filled diamonds). Data were analysed using the nonparametric Mann-Whitney test with p-values indicating significant differences after transformation of data to Log2 values.*P<0.05, ** P<0.001.

We also analysed the ratio of the main Th1 (IFN-γ and IL-12(p40)) and Th2 (IL-4 and IL-10) hallmark cytokines, however, none of the ratios of IFN-γ/IL–4, IFN-γ/IL–10, IL-12(p40)/IL-4 and IL-12(p40)/IL-10 were significantly different between patients infected with the different lineages or families (Figure 6-4).

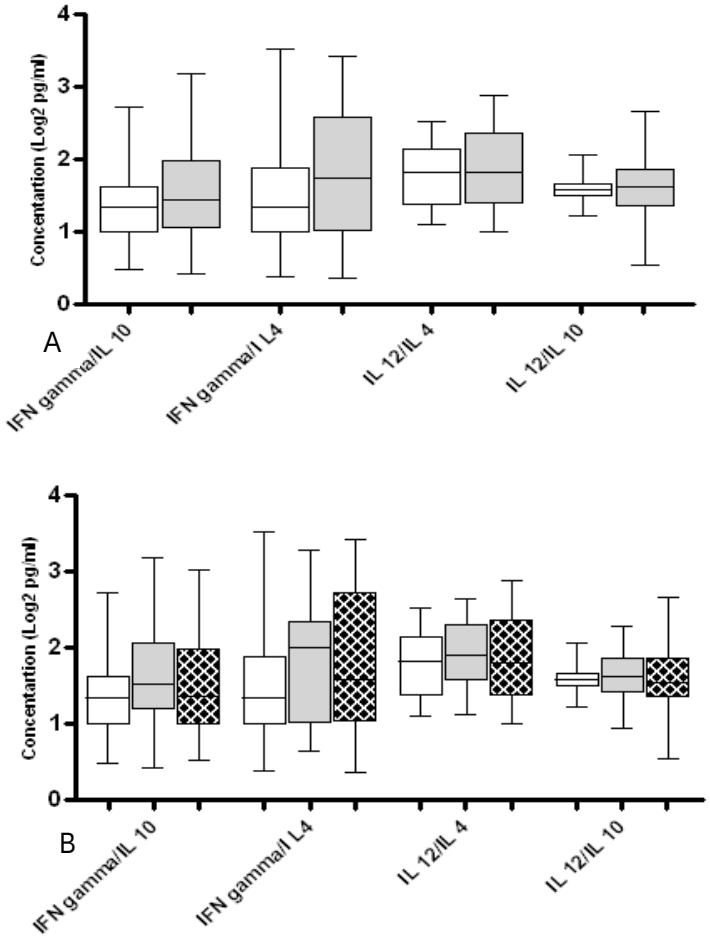


Figure 6-4 Th1/Th2 ratios of cytokines of TB cases infected with different strains.
Box plots are shown with the horizontal line indicating median levels of TB cases and the lower and upper edge of each box indicate the 25th and 75th percentiles, respectively. A) Ratio of Th1/Th2 cytokines (IFN-γ/IL-4, IFN-γ/IL-10, IL-12/IL-4 and IL-12/IL-10) of TB patients infected with lineage 3 (white bars), and lineage 4 (grey bars); B) Ratio of Th1/Th2 cytokines (IFN-γ/IL-4, IFN-γ/IL-10, IL-12/IL-4 and IL-12/IL-10) of TB patients infected with CAS family (white bars), H family (grey bars) and T families (crossed bars). Data were analysed using the nonparametric Mann-Whitney test with p-values indicating significant differences after transformation of data to Log2 values.

6.4 Discussion

Understanding the effect of the genotype of an infecting organism in the pathogenesis of tuberculosis is becoming an essential question in tuberculosis research. It is well known that T cell responses play a fundamental role during M. tuberculosis infection where a strong T cell response leads to granuloma formation and maintenance whereas a defective T cell response favours progression of disease. In the present study, we analysed the plasma level of different cytokines and chemokines in TB cases who are diseased with different M. tuberculosis genotypes, which are common in Addis Ababa, Ethiopia.

Strains belonging to the modern lineage were the only M. tuberculosis isolates circulating in the study community and we compared the plasma levels of cytokines and chemokines of patients infected with the different lineages and families within the modern lineage. Previous studies using laboratory and clinical strains have shown differences in immune response against different M. tuberculosis isolates. For example, strain NH878 has been associated with a low inflammatory immune response and increased virulence in macrophages and animal models compared to H37Rv, H37Ra, Erdman and CDC1551 (Manca et al.; 1999; Manca et al.; 2001; Manca et al.; 2005; Tsenova et al.; 2005). A recent study also showed a wide variation in the immune response after measurement of cytokines from infected human peripheral blood monocyte derived macrophages where modern lineages induced lower inflammatory responses in comparison with ancient lineages. This lower immune response might promote more rapid disease progression and increase transmission in the case of modern lineages (Portevin et al.; 2011).

In our study we compared the plasma level of 17 cytokines, which belong to proinflammatory cytokines, anti-inflammatory cytokines, angiogenic factors and

chemokines, in tuberculosis patients infected with different M. tuberculosis strains of the modern lineage. The plasma level of IL-9, IL-17, IL-7, TNF and GM-CSF was present in very low concentrations in all patients regrdless of strains. Although detectable, no significant difference in levels of EGF, fractalkine, IFN-γ, IL-1, IL-10, IL-12(p40), IL-7, IP-10, MCP-1, MCP-3, MIP-1β, and VEGF was found between patients infected with different lineages and families. We found that only the plasma level of IL-4 was significantly higher in patients infected with lineage 3 ($p<0.05$) as compared to lineage 4. We further grouped the lineages into families and similarly, it was IL-4 which showed statistical difference between the different families where patients infected with CAS family had a higher plasma level of IL-4 ($P<0.05$) as compared to patients infected with H and T family but there was no difference between H and T families.

Previous studies reported lower inflammatory responses associated with modern strains including Beijing and other strains (Lopez et al.; 2003; Chacon-Salinas et al.; 2005; Portevin et al.; 2011) where low inflammatory responses were linked to increased virulence (Manca et al.; 2004; Newton et al.; 2006). Another study in Madagascar also showed lower IFN-γ responses in tuberculosis patients and their contacts that are infected with ancient strains, like East African-Indian (EAI) strains than modern M. tuberculosis strains, like Beijing and Central Asian (CAS) strains.

Although we did not compare the two broad lineages of M. tuberculosis, ancient and modern lineage, we clearly saw a marked difference in the plasma level of IL-4 within families of the modern lineage. IL-4 is an anti inflammatory cytokine and there are hypotheses that higher level of IFN-γ and lower level of IL-4 are required for the maintenance of an effective Th1 response against M. tuberculosis. IL-4 suppresses

macrophage derived production of IL-12, thereby inhibiting differentiation of Th1 cells and inhibits cell mediated immune reactions by antagonizing the macrophage activating effect of IFN-γ. Therefore, the higher level of IL-4 in lineage 3 families might indicate that strains belonging to this family could be more virulent than the lineage 4 families of modern lineages. This present study is too small and was not designed to allow the detection of clinical differences between infections with different strains, including extent of disease, presence of cavitation and treatment response but should be investigated in future studies.

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CHAPTER 7

7. General Discussion

The immune response against M. tuberculosis is multifaceted, involving a network of innate and adaptive immune responses. Characterizing the immune response of tuberculosis, a clear understanding of the dynamics and identification of biomarkers which can discriminate between active tuberculosis disease and latent tuberculosis infection, identifying persons most likely to advance to active disease and relapse after treatment are critically needed in order to make a significant impact on and curb the global tuberculosis problem.

In the last decade or so, a lot of research has been conducted to identify biomarker(s) which may be translated into clinical and public health programs as simple and inexpensive point-of-care tests to distinguish between the different outcomes of infection and anti tuberculosis treatment (Wallis et al.; 2009; Kaufmann; 2010; Parida and Kaufmann; 2010; Wallis et al.; 2010; Walzl et al.; 2011). As part of the global effort of studying tuberculosis biomarkers, we investigated: 1) transcription of host genes in tuberculosis cases and their contacts and effect of HIV on the gene expression of co-infected cases; 2) plasma cytokine and chemokine levels of tuberculosis patients and their contacts; 3) strain diversity in the study area; and 4) the immunological consequence of tuberculosis cases infected with the different M. tuberculosis genotypes under the EDCTP and BMGF funded tuberculosis consortium projects.

Biomarker studies based on transcriptomics are one of the current research interests of different laboratories to identify genes which can discriminate tuberculosis cases and latently infected individuals, predict durable cure and indicating reactivation risk. In our study, we analysed 45 genes and the expression of IL-4d2, BLR-1, MARCO, CCL-19, IL7R, Bcl2, FcgR1A, MMP9 and LTF genes discriminate tuberculosis cases and their healthy contacts. The genes, which showed significant discriminatory expression between contacts and

tuberculosis cases in this study include molecules of the innate and adaptive immune response. Previous studies had also shown that LTF, FcgR1A, RAG33A (Jacobsen et al.; 2007), CD3e, CD8a, IL7R, BLR1, CD19, FcgR1A, CXCL10, CD4, TNF, Bcl2, MMP9, FoxP3, CASP8, CCL-4, TNRFSF1A, CASP8 and TNF (Joosten et al.; 2012) genes were differentially expressed and can discriminate between tuberculosis patients, latent infection, and uninfected people. Expression levels of RIN3, LY6G6D, C14orf2, SOCS3, KIAA2013, TEX264, ASNA1, ATP5G1, and NOLA3 identify subjects at risk for recurrent tuberculosis (Mistry et al.; 2007).

From the previously published as well as the current data there are genes that showed a similar pattern of expression in different settings and population. Although there are drawbacks in the transcriptomic platforms due to regional differences in host and microbial genetics and the lack of specificity for tuberculosis of the differentially expressed genes, the transcriptional data described above might be converted into clinically useful tools after verifying across different populations and in larger sample sizes. Across the different transcriptomic studies genes related to macrophages, B cells and T cells were identified. This expanded scope of immune paths beyond T cells is important and in any future biomarker study, due emphasis needs to be given equally to cells and molecules of both adaptive and innate immune responses.

On the other hand there are genes like FoxP3, TGFβ1 and CCL-19 that were upregulated in latently infected individuals in comparison with uninfected individuals. The upregulation of these genes could be due to the fact that this group of subjects were individuals who had recent contact with tuberculosis cases and recent infection, which may lead to limited immune activation and at the same time immune regulation to prevent immunopathology. Up-regulation of CCL-19 facilitates the recruitment of different cells. Up-regulation of both

pro- and anti-inflammatory markers in latently infected individuals who do not develop active TB during the two-year follow-up period suggests that a delicate balance between different immune phenotypes is required. A recent study reported that IL-2 induces activation and expansion of both T effector cells and Foxp3 (+) Treg populations early in M. tuberculosis infection and confers resistance against severe M. tuberculosis disease (Chen et al.; 2012).

Disease protection, progression or development of pathophysiology in tuberculosis have been linked to the type and magnitude of cytokines and chemokines that are produced, however, the specific cytokines that mediate immunologic resistance or susceptibility to mycobacteria in humans remain undefined. Cytokines and chemokines like IFN-γ (Goldsack and Kirman; 2007), TNF (Keane; 2005), IL-12(p40) (Verreck et al.; 2002), IL-17 (Khader et al.; 2007), CCL-2 (Kipnis et al.; 2003), CCL-3, CCL-4 and CCL-5 (Saukkonen et al.; 2002), CXCL-8 (Kurashima et al.; 1997), CXCL-9 and CXL-10 (Sauty et al.; 1999) have been reported to be important for control of M. tuberculosis infection.

Several studies have been conducted to identify cytokines and chemokines, which could be associated with protection or susceptibility. High level of Th1 cytokine gene expression in unstimulated PBMC (IFN-γ and IL-12) and the IL-4 antagonist IL-4δ2 were found in protected individuals in comparison with non protected individuals (Demissie et al.; 2004). Proinflammatory cytokines such as TNF, IL-12(p40) and IL-17 in 7 day stimulation of whole blood with TB antigens (ESAT-6/CFP-10 (EC), PPD or TB10.4) are increased in tuberculosis cases and can discriminate between active tuberculosis disease and latent infection (Sutherland et al.; 2010), Tuberculosis cases also have high levels of IL-8, IP10, MCP-1, and MIP-1 β in serum/plasma in comparison with non tuberculosis cases (Juffermans et al.; 1999; Azzurri et

al.; 2005; Siawaya et al.; 2009). One study adapted the commercial QFT assay, where detection of a combination of three host markers or single markers (selected from EGF, sCD40L, MIP-1•, VEGF, TGF-• or IL-1•) in stimulated or unstimulated plasma samples had promising discriminating ability between latent and active tuberculosis (Chegou et al.; 2009). In our study we measured unstimulated plasma cytokines with a panel of 17 cytokines and we found a significant discriminatory ability of plasma cytokine and chemokine levels between tuberculosis cases versus their contacts and in tuberculosis cases before and after treatment.

The type of plasma cytokines which showed a significant discriminatory power between tuberculosis cases and the contacts were Th1 (IFN-γ), Th2 type (IL-4), chemokines (IP-10, fractalkine and MCP-3) and a growth factor (EGF). The plasma cytokine pattern in tuberculosis cases showed a higher level of chemokines, an anti-inflammatory response and a suppressed proinflammatory response when compared with that of household contacts. The individual cytokines differentiated the clinical groups by themselves but it was their combination, which gave a better discriminatory power.

Although plasma cytokines are not specific to tuberculosis, from this and other previous studies directly ex vivo unstimulated plasma samples should be for considered for future study as the sample represents the systemic activation within the host but also reflects the local immune response (host markers will be released into the circulations after local production); moreover, plasma is easy to collect and does not need additional laboratory procedures. In addition, we measured the plasma level of cytokines in relation to TB treatment and we showed a significant difference in the level of several cytokines before and after treatment. Previous studies also showed a significant difference of plasma

cytokines and chemokines (Azzurri et al.; 2005; Siawaya et al.; 2009a), which strengthens the argument for the measurement of these cytokines as potential surrogate markers for anti tuberculosis treatment response.

Apart from the host genetics and environmental influences (Bellamy; 2005), the genotype of the pathogen also affects the type of immune response and the outcome of M. tuberculosis infection (Coscolla et al.; 2010). The different genotypes of M. tuberculosis are associated with specific geographical human populations and currently there are six major lineages of M.tuberculosis based on large sequence polymorphisms. Studies showed that M. tuberculosis originated in Africa and was distributed to the other parts of the world accompanying movement of the modern human (Gagneux; 2012).

We characterized 192 M. tuberculosis isolates from new smear positive tuberculosis patients in Addis Ababa using spoligotyping and all the isolates were members of the modern lineage (Lineages 3 and 4). In comparison with the phylogenetically ancient lineage, the modern M. tuberculosis lineages are more successful in terms of their geographical spread (Gagneux; 2012) and this could be the reason why all the isolates in Addis Ababa were members of the modern lineage, contrary to the historical African origin of M. tuberculosis. Members of the modern lineage are believed to be more virulent with a shorter latency in humans and this characteristic could explain the global success of the these lineages (Portevin et al.; 2011). In our study almost half of the isolates belonged to one family, the T family, which belongs to the Lineage 4 or Euro-American lineage. The T family encompasses by default strains that could not be classified in one of the families with well-established phylogeographical specificity (Brudey et al.; 2006). Hence it is frequently referred to as the "ill-defined" family of M. tuberculosis that is found worldwide.

We assessed the plasma cytokine levels of tuberculosis cases infected with the different genotypes of M. tuberculosis to test the hypothesis that immune response would be linked to the infecting genotype. There was a marked difference in the plasma levels of IL-4 between lineages of phylogenetically modern strains. IL-4 is an anti inflammatory cytokine and there are hypothesizes that suggest that higher levels of IFN-γ and lower level of IL-4 are required for the maintenance of an effective Th1 response against M. tuberculosis. The maintenance of a prolonged Th1 response against M. tuberculosis requires not only elevation of IFN-γ, but also downregulation of IL-4. There are several other studies which showed immune response differences amongst M. tuberculosis isolates where the modern lineages induced a significantly lower immune response in comparison with the ancient lineages (Portevin et al.; 2011). The present study is in keeping with this as IL-4 suppresses Th1 responses.

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CHAPTER 8

8. Conclusion

Tuberculosis continues to be a major global health problem, causing an estimated 8.8 million new cases and 1.45 million deaths annually despite the availability of a vaccine and inexpensive, effective, and reasonably well-tolerated therapy. It is true that the current available tools to diagnose, treat and protect are ineffective to combat tuberculosis. Therefore, novel intervention measures comprising rapid and reliable diagnostics, new vaccines for uninfected or latently infected people and new drugs for patients with active tuberculosis disease need to be developed to make substantial progress towards the goal of tuberculosis elimination.

Developing simple, affordable and rapid methods for detecting active cases, discriminating and diagnosing active tuberculosis disease and latent tuberculosis infection, identifying persons most likely to progress to active disease and relapse after treatment are more critical than ever before and will have a significant impact on the global tuberculosis problem. This is particularly true for developing countries where the incidence of active and latent tuberculosis is high and where smear microscopy is the only technique widely used for active case detection. A reliable test for latent infection would be valuable to guide interventions in those most likely to progress to active TB, including HIV infected people and young children.

Currently studies are on-going in many laboratories using serum, plasma, stimulated whole blood, PBMC, saliva and other samples to identify molecules which can be used as a biomarker for indicating or predicting the different clinical outcome of M. tuberculosis infection. In our study, as part of the Grand Challenges in Global Health (GCGH) consortium supported by BMGF, as well as the Africa-European Tuberculosis Consortium (AE TBC)

supported by EDCTP and with a focus on tuberculosis biomarkers, we used multiplex technologies i.e., Multiplex Ligation Dependent Probe Amplification (MLPA) technique for transcriptomics and Luminex for multiplex analysis of cytokines and chemokines. We also characterized M. tuberculosis isolates and analysed the effect of genotype on immune response by measuring plasma cytokine and chemokine levels using Luminex.

In the transciptomics study we analysed forty-five genes from whole blood sample and we found a number of genes which alone, or in combination, gave a promising discriminatory power to differentiate between tuberculosis cases and their contacts and latently infected versus uninfected individuals. Similarly, the multiplex analysis of cytokines and chemokines also showed that single or combination of plasma cytokines and chemokines discriminate the different clinical groups of tuberculosis. Major drawbacks of our studies include the use of peripheral blood samples for biomarker discovery in view of the known compartmentalization of immune responses as well as the lack of specificity of non-stimulated samples for TB as many other inflammatory conditions may induce similar responses. However, peripheral blood remains an attractive sample type due to the ease with which this sample can be obtained and unstimulated samples remain attractive as ex vivo, rapid tests have many advantages over stimulated tests that only yield results after lengthy culture periods and that require significant laboratory expertise and infrastructure.

In both the transcriptomics and multiplex study, we identified genes or cytokines, which had been reported from other studies with different designs and in different settings, thereby validating such results. The observed differences in the plasma level of cytokines and chemokines in tuberculosis patients infected with different strains of M. tuberculosis, however, emphasize the needs to take geographical factors into consideration in any future

similar work and also raise the issue that a greater focus should be given to markers that are not so dependent on infection strains.

It is clear that no single transcript or cytokine or chemokine stand out as promising biomarkers on their own but that combinations of such markers hold the greatest promise. The exact composition of such biosignatures seems to allow some flexibility and depends on the specific clinical situation, on bacterial strains that are prevalent and may also be affected by host genetic and environmental factors.